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POSSIBLE APPROACHES TO THE HEALTH EFFECTS TESTING OF FUELS AND FUEL ADDITIVES

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16. ABSTRACT

This document describes possible approaches to the testing of fuels and fuel additives for potential health effects. Such health effects testing is required of the manufacturer of a fuel or fuel additive. The health effects tests must include but are not limited to carcinogenic, teratogenic, or mutagenic effects. In order to determine the appropriate protocol for health effects testing, the following areas are discussed: (1) test materials, (2) relationship of physical and chemical properties of test materials to physiological distribution and biological activity, (3) weighing factors involved in determining an approach to appropriate health effects testing, (4) route and mode of exposure.

The possible health effects tests described are organized into the following areas: (1) toxicity (with an emphasis on pulmonary effects), (2) mutagenesis, (3) carcinogenesis, (4) teratogenesis and reproductive performance.

The final chapter describes two possible approaches to testing. This report is a technical background document and is not intended to serve as a health effects testing protocol for fuels and fuel additives.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This document addresses itself to the acquisition of data necessary for determination by the Administrator as to whether or not an increased health risk would exist should the fuel or fuel additive in question become a produce of commerce.

F. Gordon Hueter, Director
Health Effects Research Laboratory

ABSTRACT

This document describes possible approaches to the testing of fuels and fuel additives for potential health effects. Such health effects testing is required of the manufacturer of a fuel additive. The health effects tests must include but are not limited to carcinogenic, teratogenic, or mutagenic effects. In order to determine the appropriate protocol for health effects testing, the following areas are discussed: (1) test materials, (2) relationship of physical and chemical properties of test materials to physiological distribution and biological activity, (3) weighing factors involved in determining an approach to appropriate health effects testing, and (4) route and mode of exposure.

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BACKGROUND

Section 211 of the Clean Air Act of 1970 which is amended to Section 222 in 1977 by adding new subsections at the end thereof, charges the Administrator of the Environmental Protection Agency (EPA) with responsibility for the regulation of fuel and fuel additives. Registration is required by the Agency of Fuels and Fuel Additives prior to sale or introduction into commerce. The administrator shall promulgate regulations not later than August 8, 1978, to implement the authority to require the manufacturer of a fuel or fuel additive:

(A) to conduct tests to determine potential public health effects of such fuel or additives (including, but not limited to, carcinogenic, teratogenic, or mutagenic effects); and

(B) to furnish the description of any analytical techniques that can be used to detect and measure any additive in such fuel, the recommended range of concentration of such additive, and the recommended purpose in use of such additives, and such other information as is reasonable and necessary to determine the emissions resulting from the use of the fuel or additives contained in such fuel, the effect of such fuel or additives on the emission control performance of any vehicle or vehicle engine, or the extent to which emissions affect the public health or welfare; with respect to each fuel or fuel additive which is registered on the date of promulgation of such regulations and with respect to each fuel or fuel additive for which an application for registration is filed thereafter.

In addition, all such manufacturers would be required to furnish summaries of information in their possession about mechanisms of action of additives, reactions between fuels and their additives, effects of the additives on emissions, identification and measurement of emission products, and effects of the emission products on health, welfare, and emission control devices.

Additive manufacturers would be required to furnish additional information about the chemical composition and structure of additives and methods of analysis, any impurities, and sales volumes. Fuel manufacturers would be required to furnish additional information on such properties as hydrocarbon composition, polynuclear organic materials, sulfur, trace elements, vapor pressure, distillation temperatures, and cetane numbers, including methods of analysis for each of these, information on variation by geographic region, and production volume.

The registration of fuels and fuel additives is provided for in 46 CFR Part 79, A Revision of 40 CFR Part 79, (Federal Register, Volume 39, No. 46 (March 7, 1974). As revised, Part 79 designates certain fuels and additives as requiring registration: namely, motor vehicle gasolines, motor vehicle diesel fuel, and additives for use in motor vehicle gasoline and motor vehicle diesel fuels, and/or in motor vehicle crank case oil. These substances are further identified and designated in paragraphs 79.31, 79.32, 79.33 of Part 79 (46 CFR 79).

OVERVIEW

This document addresses itself to the acquisition of data necessary for determination by the Administrator as to whether or not an increased health risk would exist should the fuel or fuel additive in question become a product of commerce.

Basic to our present day culture, and to that of all economically developed areas of the world, is the generation of energy from the combustion of fossil fuels. This base supports both an advanced technology, and the mobility and livelihood of an expanding population. Whether associated with the fixed sources of industry and municipal utilities or the mobile sources of personal and commercial transportation, all practicable combustion processes result in incomplete combustion of the carbon-containing fuel. Therefore, instead of the theoretical complete combustion products of water and carbon dioxide, a complex dynamically-variant mixture of many partial oxidation products including those of other elements - such as nitrogen from the air and sulfur and similar minor component elements present in fuel - as well as those of carbon, hydrogen, and oxygen, is actually produced. The exact composition of the combustion products varies with the characteristics of each individual fuel, combustion conditions, environmental conditions, etc. Because the combustible fuel substances are all natural products, it is not possible to control their chemical composition or the attendant composition of the combustion products.

Urban areas provide combinations of: (1) relatively high atmospheric concentrations of emissions from multiple-source combustion processes; (2) a man-made environment which restricts the dissipation of these emissions; and (3) large numbers of people who have to live and function in this environment. In this situation, motor vehicle emissions represent a major source of concern with respect to the health and well being of human populations. Consequently, a significant research effort has been directed toward understanding health effects implications of emission components and whole emissions. From the results of such studies, it is possible to correlate certain specific biological effects with the occurrence and concentration of specific chemical entities in the emissions. Such knowledge is extremely valuable, but the current data base falls short of enabling predictions of the health effects of lifetime exposures to emissions for heterogeneous human populations.

However, it is now necessary to consider, (1) whether a given fuel composition will have more or less, or better or worse, health effects than another fuel composition; and (2) whether the addition of single or multiple optional ingredients, defined here as "additives", could possibly alter the health effects associated with the emissions in a manner which would result in increased hazard to human populations. These optional additions to, or alterations in, the basic hydrocarbon fuels consist mainly of chemicals selected for the purpose of improving specific aspects of engine performance, fuel stability, emissions characteristics, etc. As such additives are synthetic chemicals with inherent manufacturing costs, economic considerations dictate that they will be used sparingly in high-volume bulk fuels. The very low concentrations of these iptional additive substances along with certain physical chemical properties pertinent to the choice of chemical nature of such additives (e.g., low volatility) all contribute to predictions of generally low levels of atmospheric contamination by either the combustion products of such additives or the unburned additive material itself in the exhaust gas stream. In fact, at normal use levels for some of these additives, conventional analytical methods are not able to detect any residual unburned additive compound in the emissions, while concentrations of specific combustion products resulting from these same additives may be extremely low.

However, conventional bulk analyses may not reflect biologically effective concentrations as there has been found to be a surface predominance of toxic trace elements in fine particles produced by combustion processes, with an inverse relationship between particle size and trace element concentration. This inverse relationship is thought to be due to the volatilization of trace elements or their compounds followed by condensation onto particle surfaces as the temperature drops (Linton _et al_., 1976). Polyalkyl gasoline additives and reactions between ammonia, nitric oxide and soot have both been linked to the occurrence of organic nitrogen-containing bases on the surfaces of filter-collected particles.

The existence of such low-dose levels does not necessarily mean that human systems do not respond, but rather that resulting effects are likely to be lost among the general variability of measureable responses. Low-dose response measurement and interpretation is immensely complex and must be considereded within the context of synergistic and potentiating effects, sub-toxic effects, bioaccumulation, biomagnification, and many other factors related to variability in health status of specific sub-groups within the human population.

For motor vehicle emissions, the primary human target systems are most likely to be the external organs of eye and skin and the respiratory system. Exposure of eye and skin are generally associated with transient irritative effects, while insult to the respiratory system has much more serious implications over a much wider temporal scale. For example, gaseous emission components which cause irritation of eyes and skin will also cause inflammation of tissues in the respiratory tract, while the properties of the gases (specifically, solubility and volatility) will determine the depth of penetration into the respiratory tract, and consequently the nature of the symptoms produced.

In addition to possible overlapping effects of individual gaseous components of emissions upon human respiratory systems, the particulate phase may also exert specific toxic effects - the site of which will be largely determined by the physical characteristics of the particulate. For example, size and shape will determine deposition site and clearance mechanism; while the hygroscopicity, partition coefficient, and solubility in lung fluids will determine particle growth and aggregation, lung tissue uptake and binding, and retention time. Moreover, one pollutant may influence the biological effect of another; for example, normal respiratory clearance mechanisms for particulates could be inhibited by gaseous components of emissions, thus contributing to increased retention times for emission particulates and increased retention of inhaled microorganisms along with diminution of normal microbicidal capacity. These effects may result in both exacerbation of particulate toxicity and increase in susceptibility to infection.

In addition to rapid absorption of inhaled organic compounds by respiratory tissues, there is a marked concentration of basic lipophilic amines by the lung, even when the route of administration is other than inhalation (Junod, 1974; Philpot _et al_., 1977). This chemical class includes polyalkyl amine fuel additives and potentially carcinogenic aromatic amine combustion products - precisely those nitrogen-containing organic bases found to be concentrated on the surfaces of respirable smog particles (Novakov _et al_., 1972).

Not only are certain hydrophobic substances absorbed or concentrated by lung tissue or localized in the respiratory tract by deposition, but lung and respiratory tract tissues possess a remarkable metabolic capacity as well; in fact, for some chemical classes, lung is more active metabolically than liver. For example, lung has almost 50 times the metabolic capacity for N-methyl indoleamines as liver (Brown, 1974); and although liver mixed function oxidase activity is much higher than that of the lung, some substrates such as benzene, decane, benzphetamine, biphenyl, and N-methyl-p-chloroaniline are metabolized by lung microsomes at rates equal to or greater than those obtained with liver microsomes. In fact, p-xylene is hydroxylated 4 times faster by rabbit lung microsomes than by rabbit liver microsomes on a weight of tissue protein basis and 16-20 times faster on a mole of enzyme basis (Philpot _et al_., 1977). Since metabolism of xenobiotic chemicals involves a cluster of interrelated enzymes the number and specificity of which apparently varies from organ to organ, the overall pattern of metabolites produced by a given tissue may well be the determining factor in target organ carcinogenesis. Metabolites produced in the lung may be either returned in the lung or distributed to other organs of the body via the circulation, hence lung metabolic capacity assumes unique importance.

There are still other types of chemical structure for which lung is the target organ for toxic effects regardless of route of exposure; nitro-olefins are such a class, specifically, the automotive emission constituent-olefins C_2 to C_7 which react with atmospheric NO_x to produce nitro-olefins (Deichmann et al., 1958).

All of these factors are relevant to the biological effects of inhaled motor vehicle emissions. For example, if the particulate phase of emissions from a specific fuel, or fuel plus additive combination, is of a roughly spherical shape and diameter less than three microns, the particulates will be preferentially deposited within the alveolar regions of the lung where clearance processes can have half-lives of months to years for relatively insoluble particles (Casarett, 1975; Kilburn, 1977). The numbers of such small particles retained in the lung from a single exposure may be quite large (Lippmann, 1977); therefore, if exposure conditions are chronic, it is reasonable to expect that the mass of such material will continue to accumulate. It is not unreasonable to anticipate a situation in which a biologically-active trace component of emissions could be absorbed upon the considerable surface area of relatively insoluble particulates less than three microns in diameter. Therefore, upon inhalation, these particles would be deposited in the alveolar regions of the lungs subject to clearance by alveolar macrophages and by dissolution in lung fluids, and other mechanisms. However, if the absorbed material is either cytotoxic to macrophages, or insoluble in lung fluids, or both, it would resist clearance by either Phase III or IV mechanisms and would continue to accumulate over long periods of time.* In this, or a similar situation, even the most minute quantities of any given chemical species take on a significance quite out of proportion to its existence in barely-detectable concentrations in whole emissions; particularly if the chemical species under consideration acts as carcinogen, cocarcinogen, or tumor promoter, initiating irreversible delayed toxic processes (cancer) with lead times of five to thirty years.

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*FOOTNOTE

> "Phase I clearance rates, with half-times of minutes to hours, reflect clearance of particulates from the upper respiratory tract and the naso-pharyngeal regions. Phases II and III both involve alveolar macrophages, but whereas Phase II phagocytic clearance rates have half-times of the order of 2 to 6 weeks, Phase III half-times are on the order of months and are considered to involve sequestration of materials. Phase IV clearance when it occurs, is thought to depend entirely on solubilization, and, depending upon the chemical nature of the substance, may exhibit half-times of months to years (Casarett, 1975)."

In other words, no matter how low the concentration per unit time reaching the target biological system is, if the normal turnover mechanisms of the body have not adapted or evolved to handle this type of exogenous substance, it will accumulate. Since dose is a product of concentration and time, the actual dose of trace contaminant to which the target tissue is exposed may become quite significant under conditions of chronic exposure. For this reason, there is a real need for the development of screening tests capable of predicting chronic health effects without requiring chronic animal exposures.

There are other conditions under which a trace component, or even an occasionally occurring trace component of emissions could possess biological significance disproportionate to actual target dose level. Such a chemical entity could act as inhibitor or modulator of key enzymes, or interact with vital hormones or hormone receptors. The physiological level of these substances is generally in the nanomolar to picomolar (10^{-9} to 10^{-12} molar) range or below, so low concentrations of a modulator substance might be quite effective. Another possible role for a trace toxicant could be as non-enzymatic catalyst of a nonphysiological detrimental reaction; similarly, a trace pollutant could non-enzymatically trigger a physiological cascade-type reaction producing biologic amplification analogous to the complement system of blood.

A concept of relevance to the present situation involves consideration of human exposure to low levels of environmental carcinogens in terms of life-shortening (Albert and Altshuler, 1976). Epidemiological evidence suggests that as much as 80% of human cnacer is due to exposure to environmental carcinogens. This incidence of cancer is considered to represent a background or baseline of environmentally-caused cancer. The introduction of a cocarcinogen, or an increase in the concentration of an existing carcinogen may shorten the time-to-tumor appearance, thereby increasing the incidence and shortening the lives of affected individuals (WHO-IARC, 1976). The impact of the new carcinogen can be evaluated from tumor incidence measurements in animals, and related to the human condition.

Some element of public health risk associated with exposure to combustion products is unavoidable because of the overriding need for energy generated in the combustion process. In order to determine whether a given fuel or fuel additive would present an undue health risk, it will be necessary to make judgments based upon: 1) whether its use would result in an identifiable incremental increase in risk; 2) whether the increased risk is counterbalanced by a worthwhile benefit.

The most logical way to approach an estimate of possible increased public health risks appears to be by comparison of the biological effects of the combustion products of a given fuel or fuel additive with those effects resulting from the combustion products of a standardized reference fuel and specified engine system. In formulating these recommendations for drafting of protocols for health effects evaluation, consideration has been given to the following reports:

(1) PRINCIPLES FOR EVALUATING CHEMICALS IN THE ENVIRONMENT

National Academy of Sciences
Washington, D.C., 1975

(2) FUELS AND FUEL ADDITIVES FOR HIGHWAY VEHICLES AND THEIR COMBUSTION PRODUCTS

National Academy of Sciences-National
Research Council, 1976

(3) Report - Working Conference on Health Intelligence for Fuels and Fuel Additives, 1973.

Convened by: Fuels and Fuel Additive Registration Program, National Environmental Health Research Center - Environmental Protection Agency, Research Triangle Park, North Carolina.

These scientific panels have unanimously stressed the importance of performing health effects testing of fuels and fuel additives under conditions which are as realistic as possible. These conditions would include: exposure route, biological target systems, engine operation, realistic atmospheric concentrations of combustion products, and the full range of atmospheric conditions such as irradiation with light of from 300 to 700 nanometers, the absence of such irradiation, varied humidity conditions, etc.

Several of these advisory groups (NAS, 1975; NAS-NRC, 1976; NEHRC, 1973) have called attention to the necessity for priority attention to be given to effects of subchronic and chronic exposures. Exposures of this nature implicate such health effects as respiratory infections, pulmonary diseases, and possible irreversible, delayed effects such as carcinogenesis, mutagenesis, or teratogenesis.

It should perhaps be pointed out here that human populations *will* actually be exposed to the subject fuels and fuel additives and their emissions products in the atmosphere, since the pertinent legislation permits these substances to be registered, marketed, and used during the period in which health effects testing will be performed.

The scientific panels mentioned above recommend that health effects testing must employ *in vitro* and selected whole animal studies, as a preliminary screen of biological activity, in an effort to limit the cost and time required per test material. However, it is also emphasized that whole animal exposures which are carefully designed and controlled should be used to confirm screening bioassays.

HAZARD EVALUATION

The projection and assessment of hazards to human health which could result from the widespread use of a fuel or fuel additive requires a great deal of specific data.

A. Preliminary estimate of exposure

In order to make a preliminary estimate of exposure of human populations, it will be necessary to obtain data on:

(1) quantities of the substance to be released to the environment as a result of production, use, and disposal;
(2) physical and chemical properties of the substance;
(3) the environmental transformation of the substance;
(4) likelihood of bioaccumulation.

B. Refinement of exposure estimate

In order to refine and improve the estimate of exposure, it will be necessary to obtain a great deal of more specific data for each fuel or fuel additive; specifically:

(1) physical and chemical properties, biological activities of the products in all forms - i.e., active ingredients, commercial formulation, formulation in fuel;
(2) physical and chemical properties and biological activities of products after combustion, both in the presence and absence of irradiation by light of wave lengths reaching the atmosphere;
(3) mechanisms and rate of appearance of original or altered product in combustion products, specifically: a) how much of substance comes through engine unchanged (survival index), b) mechanisms and rates of formation of combustion products (plus or minus irradiation) - i.e., whether the rates of product appearance are determined by chemical kinetics or by physical conditions of the engine combustion process;
(4) quantitative estimation of concentrations likely to be delivered via all possible routes of human exposure including: a) inhalation, the most likely route of exposure for vapors of unburned

material or material which goes through the combustion process unchanged, or combustion products; b) skin absorption and eye contact due to consumer product use, accidental spillage, or waste disposal; c) ingestion due to environmental contamination.

I. TEST MATERIALS

Since human exposure to fuels and fuel additives may occur from both the initial chemical forms and the combustion products, and since both states of matter may or may not be subjected to irradiation in the environment, the potential health effects of all forms of these substances must be considered for their most likely routes of exposure. Therefore, at the discretion of the Administrator, testing for specific health effects of any or all of the forms of registered fuels, fuel additives, or fuel/fuel additive combinations described below may be required.

A. Uncombusted State

1. Fuels

"Fuel" means any material which is capable of releasing energy or power by combustion or other chemical or physical reaction.

a. Motor Vehicle Gasoline

The types of gasoline being designated are: unleaded; leaded, premium; and leaded, nonpremium.

b. Motor Vehicle Diesel Fuel

The designated grades of motor vehicle diesel fuels are: grade 1-D and grade 2-D.

c. Motor Oil

Motor vehicle engine oils employed to lubricate cylinder walls and intake and exhaust valves of both standard piston and rotary engines are also defined as fuels in that a portion of the oil is in fact combusted in the normal operation of the engine.

2. Additives

"Additive" means any substance, other than one composed solely of carbon and/or hydrogen, that is intentionally added to a fuel named in the designation (including any added to a motor vehicle's fuel system) and that is not removed prior to sale or use.

a. Individual Ingredients

Individual ingredients are those separate chemical substances incorporated into the additive product of commerce:

(1) purified - individual ingredients subjected to chemical purification;
(2) technical grade - individual ingredient chemical substance resulting from chemical manufacturing processes without subsequent purification.

b. Commercial Formulation

The additive product of commerce including all principal and minor ingredients such as those listed in (1) and (2) above.

3. Combinations

a. Fuel Plus Single Additive (or Additive Package)

b. Single Fuel Plus Multiple-Additive Mixture

c. Multiple-Additive Mixture

B. Combustion Products

There appears to be a consensus that the problems of fuel and fuel additive toxicology must include consideration of whole exhaust effluent (p. 8, references (1) and (3)). This approach would compare the toxicity of emissions from a reference fuel with those from the same fuel plus additives (included at the concentration prior to combustion specified by the manufacturer) or with a test fuel. It is anticipated that the engine design, emissions control accessories, fuel composition range, engine operating conditions, conditions of irradiation, and other pertinent variables would be specified by the Administrator for each additive in accord with current and future ambient Air Quality Standards.

The whole exhaust effluent, irradiated or non-irradiated, represents the most relevant test material for study of effects of alterations in fuel composition upon health effects associated with motor vehicle emissions. However, for many of the accepted screening tests for biological activity, it will not be practical to employ whole exhaust, but components, i.e., particulates and isolated fractions can be tested. To some extent, the actual form of test material employed may be dictated by requirements and limitations of the test selected. Therefore, to attempt to correlate biological effect with additive presence, it will be necessary to determine the fate of the additive upon combustion; specifically, with what physical phase(s) or processed fraction(s) of the emissions is either the unreacted additive, its combustion products or both associated (Bradow and Sigsby, 1976)?

1. Whole Exhaust

The utilization of whole exhaust as a test material is suitable for whole animal exposures if allowance is made for cooling of the hot emissions and for diluting with air so that non-toxic levels of carbon monoxide are obtained. Systems for the generation and control of whole exhaust suitable for toxicologic studies have been described.

Facilities for the generation of exhaust emissions representative of that produced under actual motor vehicle traffic conditions, and for the monitoring, irradiation, temperature control, chemical and physical characterization, and delivery of these emissions to biological test systems require a considerable investment of funds. Such facilities are of limited availability for health effects testing.

The irradiation of whole exhaust significantly alters the concentrations and character of the chemical species present and causes a marked increase in irritant effects. For those experiments involving irradiated exhaust, it is important that the exhaust effluent be exposed to light containing wave lengths from 280 nanometers to 700 nanometers, immediately upon exit - i.e., simultaneously with or prior to dilution.

2. Characterized Emission Products

The individual components of exhaust emissions may be described on the basis of either their physical properties or chemical properties. For those test situations in which whole, freshly generated exhaust cannot be employed, it may be advisable to test all collectible or isolatable fractions. A given additive could alter the emission from a specific fuel in a variety of ways. For example, (1) additive combustion products may be associated both with particulate and/or vapor phase; (2) unburned additives may be adsorbed on particulates; (3) the presence of additives may alter the size of particulates formed; and (4) the presence of additives may alter the chemical nature of species in either, or both, vapor and particulate phase.

If the emissions generated from fuel containing a specific additive are determined upon chemical analysis to be the same as obtained from the same fuel in the absence of the additive (i.e., unchanged by the additive), no further testing is necessary. If the additive or its combustion products are definitively determined to exist solely in either the vapor phase or the particulate phase, that phase with which the additive is associated could be used as the biological test material. It may be deemed appropriate by the Administrator to screen for certain health effects on the basis of either the physical properties - i.e., vapor phase versus particulate phase - or the chemical properties - for example, oxygenated hydrocarbons, polycyclic hydrocarbons, acidic extracts, etc.

a. Physically Isolated Phases

(1) Vapor Phase

Exhaust gas contains low molecular weight hydrocarbons, some of which are present in the original fuel and others that are not. Specific hydrocarbons which are known to occur in emissions include methane, ethane, ethylene, acetylene, propylene, four-carbon (C_4) olefins, and sometimes propadiene and methyl acetylene. These low molecular weight hydrocarbons may constitute 40-60% of the volume of the vapor phase. In addition, fuel components include hydrocarbons heavier than butane and may number over 100 different hydrocarbons. The hot exhaust also contains vaporized aromatic hydrocarbons such as ethylbenzene, styrene, benzene, benzaldehyde, and toluene. In addition, oxygenated organic compounds such as aldehydes, ketones, alcohols, ethers, esters, acids, and phenols are found in exhaust vapor. The total oxygenate concentration is about one-tenth the total hydrocarbon concentration. There are also epoxide and peroxide compounds, which have apparently not been well characterized in exhaust, but which might be particularly significant in terms of health effects. In addition to the organic compounds, the vapor phase of emissions may also contain oxides of carbon, sulfur, and nitrogen; other sulfur compounds; other nitrogen compounds; and oxidant species.

Reference material dealing with the composition, analysis, and sampling of motor vehicle vapor phase emissions are provided in the references at the end of this section.

(2) Particulate Phase

Motor vehicle emissions contain particulates which result from both primary emissions and secondary reactions. In general the primary particulates are below 0.08 microns, a size range described as "Transient Nuclei Range" (or Aitken range). Secondary particulates formed from the condensation of emission products in the atmosphere range from 0.08 microns up to approximately 1 to 2 microns, a range known as the "Accumulation Range." The range from approximately 1 to 2 microns, with a maximum of 6 microns, is known as the "Mechanical Aerosol Range" and includes particles produced by mechanical processes. The polycyclic aromatic hydrocarbons of emissions are primary particulates as are a variety of metal particulates. A number of secondary particulates result from gas-to-particle conversion processes occurring in emissions-containing atmospheres (Finlayson-Pitts and Pitts, 1976).

The biological effects of particulates obtained from both irradiated and nonirradiated whole exhaust should be considered.

b. Chemically Identifiable Fraction

For the purposes of these Guidelines, a chemically identifiable fraction of whole test exhaust (irradiated and nonirradiated) may be defined as:

(1) a single chemical compound established to be a combustion product of experimental emissions (reference fuel plus additive, reference fuel plus test fuel, etc.) which is absent in control emissions (reference fuel only);
(2) a physically or chemically derived fraction or extract of both experimental emissions and control emissions.

References describing the preparation of such chemically identifiable fractions are included in those listed at the end of this section.

3. Additive Pyrolysis Products

In certain cases, additive pyrolysis products may be a suitable preliminary test material. These should be generated in an air atmosphere combustion chamber of a high-vacuum system with provision for immediate trapping - either cryogenic or absorption (Axelrod and Lodge, 1976) - or filtration and trapping.

I. B. References for Test Materials - Combustion Products

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I. B. References for Test Materials - Combustion Products (continued)

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I. B. References for Test Materials - Combustion Products (continued)

2. Characterized Emission Products (continued)

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II. RELATIONSHIP OF PHYSICAL AND CHEMICAL PROPERTIES OF TEST MATERIALS TO PHYSIOLOGICAL DISTRIBUTION AND BIOLOGICAL ACTIVITY

Exposure via the pulmonary route to a pollutant mixture such as motor vehicle emissions is influenced markedly by a complex interplay of variables relating to pollutant-component physical and chemical properties, respiratory tract physiology, and host defense mechanisms. Any alteration in properties of an emission component(s) due to the inclusion of additive, or to the substitution of a test fuel for the reference fuel, could result in physiologically significant alterations in interactive mechanisms. Some of the pollutant physical and chemical properties considered most relevant to actual human inhalation exposures to fuel and fuel additive test emissions are outlined here.

A. Physical Properties

Relevant physical properties of particulate pollutants are size, shape, electrical charge, and a function known as aerodynamic diameter (a parameter which incorporates both particle density and drag, and can be described as the diameter of a unit density sphere having the same terminal settling velocity as the particle in question, whatever its size, shape, and density) (Lippmann, 1977). Size of the particle serves as a general determinant of site of deposition within the respiratory tract in that particles greater than 10μ are generally screened out in the nasal passages, while only particles smaller than 3μ reach the deep lung or alveolar region. Shape of the particle is significant in that fibers of narrow cross section but long length may reach much deeper regions in the respiratory tract than would otherwise be expected. For example, asbestos fibers 200μ long have been observed in human lung samples (Lippmann, 1977). Particles with electric charge can exhibit enhanced respiratory tract deposition resulting from image charges induced on the surface of air waves by the charged particles. Aerodynamic diameter is the most appropriate parameter in terms of particle deposition by impaction and sedimentation processes which usually account for most of the deposition by mass in the head and lungs (Lippmann, 1977). Aerodynamic size can be measured directly for particles using an aerosol spectrometer.

Physical chemical properties affecting respiratory tract deposition and retention are: hygroscopicity, solubility in various lung fluids, partition coefficient, and quantity and nature of adsorptive surfaces of particulates. Hygroscopic particles may aggregate or swell within the moist environment of the respiratory tract producing a significant change in effective particle size.

B. Chemical Properties

Highly soluble gases and other materials affect primarily the upper respiratory tract; examples are ammonia, alkaline dusts and mists, hydrogen chloride, hydrogen fluoride, and sulfur dioxide. Compounds of intermediate solubility affect both upper respiratory tract and pulmonary tissue (such compounds include ozone, oxygen, nitrogen dioxide, halogens, diethyl or dimethyl sulfate, and phosphorus chlorides). However, certain insoluble materials (i.e., insoluble in aqueous media) such as ethyl ether and many organic vapors, partition so readily into the lipophilic tissues of the alveolar regions that they do not accumulate there, but can accumulate in the upper respiratory tract and bronchi to produce irritation (Casarett, 1975).

The solubility of so-called "insoluble" particles in lung fluids is an extremely important factor, in that many organic polycyclic aromatic hydrocarbons or metallo-organic compounds may be quite insoluble in water, but be solubilized appreciably by lipid surfactant materials, mucus secretions, or the serum fluids of the alveolar regions of the lungs. For example, polycyclic aromatic hydrocarbons are readily eluted from soot by serum proteins (Falk _et al_., 1958). It has been demonstrated that the clearance half-times of many insoluble dusts in the lung are proportional to their solubilities in physiological fluids (Kilburn, 1977).

C. Chemical Structure

An extensive scientific literature relating variation in chemical structure with variation in biological activity exists for the fields of medicinal chemistry, pharmacology, and physical chemistry. A number of physicochemical parameters such as partition coefficient, the Hammett constant, a charge transfer constant, and chromatographic hydrophobic parameters have been employed to compare biological activity of structurally analogous or similar compounds. In addition, such parameters can be employed to predict skin permeability, cellular uptake, bioconcentration and similar effects based on the partition coefficient (octanol-water system) or specific lipid solubility. Many of the potential test substances considered herein contain chemical groupings or structures amenable to some degree of predictive analysis by these methods with respect to biological retention, distribution, and activity. For those test substances for which chemical structure or chemical class is known to the manufacturer, the scientific literature may provide information on toxicity of the test substance or structurally similar compounds.

The most important parameters related to chemical structure and chemical properties for all pollutants deriving from fuels and fuel additives are considered to be:

(1) partition coefficient in octanol-water (Hansch and Elkins, 1971);

(2) aqueous solubility (Hague and Schmedding, 1975), preferably at 37°, and pH of aqueous solution;
(3) chemical structure (if it is not possible to determine chemical structure, then minimal chemical characterization should include as complete a description as possible, e.g., aromatic or polycyclic hydrocarbon compounds containing carbonyl oxygen, or cyclic nitrogen-containing ring structures); and
(4) lipid solubility (preferably at 37°).

The chemical parameters listed above apply to all physical states of test materials - i.e., liquid, vapor, and solid. Additionally, if the test material is particulate in nature, it will be most important also to characterize it as to certain physical properties, specifically:

(5) size and shape;
(6) aerodynamic diameter (Lippmann, 1977); and
(7) hygroscopicity.

It would also be extremely valuable to determine the nature of substances adsorbed on the surfaces of particulates as this is a mechanism for concentrating and presenting to the respiratory system potentially toxic chemical species formed or volatilized during combustion (Linton *et al*., 1976; Novakov *et al*., 1972).

III. WEIGHTING FACTORS INVOLVED IN DETERMINING AN APPROACH TO APPROPRIATE HEALTH EFFECTS TESTING

The dimensions and complexity of the task of evaluating the impact of candidate fuels and fuel additives upon human health dictate a need for flexibility of approach and a steady flow of informed, knowledgeable decision making. The most important decisions in the entire scheme of evaluation will be the choice of which fuels and fuel additives to test. Guidance for such decisions must come from as detailed an estimation of human exposure conditions as possible. For a given fuel or fuel additive, the factors outlined here should be taken into consideration in the selection of both test material and test approach.

A. Amount of Raw Material Produced

The quantities of material involved in production, use, and disposal constitute the limits of potential human exposure. These data may be obtained from chemical industry bulk production volumes and volume by use classifications.

B. Amount of Potentially Toxic Material Emitted

Data obtained according to the Emissions Characterization Protocol (Bradow and Sigsby, 1976) and from tests performed by EPA and other laboratories should be assessed in making estimates of the amount of potentially toxic material which may be introduced into the environment.

The amount of total toxic material emitted is an important first consideration. However, it must be taken into account that if fuel additives do indeed survive the combustion process, they could be volatilized and condense on the surfaces of particulates as they form, resulting in essentially the total bulk volume of additive being localized and concentrated on the surfaces of respirable particles. In addition to the increase in effective environmental concentration, the additives are in an optimal form for presentation to human respiratory systems.

Taken together these data will indicate the potential for biological activity and the likely deposition sites and retention times for both human targets and the environment.

C. Persistence and Chemical Form

The degree of persistence and the chemical form of material which persists become significant factors in considering human exposure to fuel additives. An ideal approach to evaluating this factor for a given additive would be to attempt a mass balance in which a given charge of additive to an engine is followed through the environment. However, for numerous reasons this is not a practicable approach. Estimation of the basic processes involved in environmental transport and assimilation, with subsequent extrapolation, appears preferable.

1. Persistence in the Environment and Biological Organisms

Alterations within the environment may proceed through chemical, photochemical, and microbial processes.

2. Bioaccumulation

The potential of a given additive for bioaccumulation can be estimated from its partition coefficient and its tendency to form complexes with sulfhydryl and amino groups (NAS-NRC, 1975).

3. Biomagnification

The potential of a substance for biomagnification would involve measurement of bioconcentration factors for food chain organisms. Aquatic organisms are able to concentrate (perhaps 10^5 to 10^6 fold) some lipophilic organic compounds and heavy metals into their tissues (DHEW-NIEHS, 1977).

4. Biotransformation to More Toxic Forms

Transformation of a substance by biological systems (i.e., microorganisms) to a more toxic chemical form should be taken into consideration. The biotransformation of mercury to the more toxic methyl mercury in the environment is an example of the importance of this factor.

D. Evaluation of Existing Health Data

A search and evaluation of the scientific literature pertaining to experimental and epidemiological data relevant for the actual exposure agent itself (if known) and for analogous chemical structures, emissions fractions, etc., should be performed. This search and evaluation should include both the original test substance itself as well as its combustion products, also the potential biological activity of any chemical substituent groups, or structural moieties. In addition, any information available on environmental persistence or transformation, both chemical and biological, should also be considered. This evaluation should include weighting of the scientific evidence as to quality of data, adequacy of data, and the kind of response induced.

E. Risk Assessment Analysis

The objective of the most thorough and comprehensive of testing schemes and collection of epidemiological information is the assessment of risk to human populations. If at all possible, input to this assessment process should include both human data and a range of experimental results. However, for the present problem of risk assessment attendant to comparison of exposure to exhaust emissions from a fuel with an additive versus those from a fuel without an additive, human data may be difficult to obtain.

The potential for cumulative, synergistic, or potentiating effects resulting from low-dose chronic exposures may be a complicating factor in risk assessment analysis for fuel and fuel additive health effects. Other complicating factors in this particular risk assessment analysis involve the superimposition of any test emissions effects upon: pulmonary disease states like emphysema; metabolism of polycyclic aromatic hydrocarbon compounds from tobacco smoke; effects of other inhaled pollutants; aging; and genetic factors (e.g., alpha-1-antitrypsin deficiency, a familial deficiency of a key protease inhibitor which predisposes affected individuals to emphysema at an early age). Population groups into which these factors segregate may be susceptible to effects of any exhaust emissions, and it may not be possible to evaluate an increase in susceptibility due to the inclusion of additives.

For the purposes of risk assessment, a toxic response observed in animals (more than one species) should correlate with a corresponding human health effect, otherwise extrapolation between species is meaningless. A primary concern for human exposure to fuel and fuel additive test emissions is induction or enhancement of pulmonary carcinogenesis or cocarcinogenesis.

Once a background of acceptable quality animal response data for a given additive is obtained, extrapolation for purposes of estimating low effect levels for humans and thereby defining exposure limits can be undertaken. There are several mathematical models employed for this type of extrapolation; these models are of two basic types:

(1) dichotomous response models and
(2) "time-to-occurrence" as a function of dose level models.

Dichotomous models include the "one-hit" model and the Mantel-Bryan probit model; while examples of time-to-occurrence models are the lognormal and the Weibull (Peto *et al.*, 1972). Factors involved in the applications of these various models for risk estimation have been considered in depth, and recommendations made as to interim procedures for setting levels of qualitatively unavoidable chemicals in the environment by the Subcommittee on Estimation of Risks of Irreversible, Delayed Toxicity to the DHEW Committee to Coordinate

Toxicology and Related Programs (Hoel et al., 1975). Additional background discussion is provided by "Biological and Statistical Considerations in the Assessment of Risk", Chapter V, in reference NAS-NRC, 1975. A valuable discussion of problems attendant to extrapolation of toxicity data from animals to man has also been presented recently (Dixon, 1976).

IV. ROUTE AND MODE OF EXPOSURE

For new motor vehicle fuels and fuel additives, the major route of human exposure will be by inhalation of air containing emission products. These emission products may or may not have been irradiated, depending upon time of day and weather conditions; they will also be subject to a number of other environmental variables. All of these factors will have a direct bearing on the nature and range of health effects which could result from inhalation of emission containing atmospheres, and from exposure of eye and skin.

There are other routes of human exposure which also should be considered although they are anticipated to be infrequent and of low magnitude compared to the inhalation of emissions. For example, for unburned fuels, additive ingredients, additive formulations, and fuels containing additives, human exposure may take place through: (1) inhalation of vapors, (2) splashing onto skin and into eyes, or (3) ingestion due to environmental contamination. Photochemical reactions may or may not occur for all of these exposure conditions.

The considerations outlined in this section apply principally to whole animal toxicity testing; however, *in vitro* systems most relevant to each route of human exposure are indicated.

A. Pulmonary

1. Inhalation

For whole animal testing of fuel and fuel additive health effects, the importance of exposure by the pulmonary route and specifically, the inhalation mode, cannot be overemphasized. Inhalation toxicology is considerably more complex than oral toxicology, and it is essential that investigators demonstrate knowledge of and capability in the application of inhalation toxicology. More so than for any other route of exposure, the chemical and physical properties of the specific individual test agent will determine: the design of the system for generating and metering the test atmosphere; design of the exposure apparatus; dose estimation and calculation; exposure schedule; and the nature of the biological effects produced. The interrelationships of the properties of the toxicant with the nature of biological response are outlined in Section V. The significance of the properties of the toxicant with respect to exposure methodology and inhalation toxicologic assessment is discussed in a number of references from the scientific literature presented at the end of this section.

Assessment of the health effects of fuel and fuel additives emissions will necessitate inhalation exposure to atmospheres containing particulates. Considerations which are basic to the performance of such tests include: behavior of aerosols; aerosol characterization; pulmonary deposition; particle clearance by the lungs; and pulmonary responses. Aerosol behavior is determined by factors such as particle size, particle size distribution, sedimentation rates, coagulation, and diffusion. For aerosol characterization, the most commonly measured parameters are particle size and mass. As a general rule, the toxicity of inhaled materials is most considerably related to the mass of material inhaled and the number of particles inhaled, measured as the number of particles per unit volume of air. Particle number is also occasionally directly related to the effect observed, as is particle surface area; however, the distribution of the mass of material is more frequently relatable to toxic effect. In terms of methodology, size or mass frequency distributions are the most complete, reproducible, and acceptable means of measurement and expression of particle size. A knowledge of particle clearance pathways and rates is necessary for selecting the proper exposure schedule and duration.

Pulmonary deposition of particles, particle clearance, and pulmonary responses are dealt with in Section II.

In addition to a wide variety of specific lung effects, the administration of a toxicant by inhalation could result in as many systemic effects as toxicants given by the oral route. The systemic effects may differ significantly between the two routes because of different metabolites formed by the lung and by the liver, or the magnitude of the effects may differ for a number of reasons such as different clearance mechanisms.

Test materials appropriate for administration to whole animals via the inhalation mode include: whole exhaust (either fresh or aged, and with or without irradiation); volatile exhaust fractions; or aerosolized fractions of whole exhaust or chemically-characterized fractions.

2. Intratracheal Instillation

In some cases, intratracheal instillation may be the most logical mode of exposure for the pulmonary route. For example, in cases where the amount of available test material is very limited, or is known to be very hazardous (such as contain a known carcinogen), this would probably be the exposure mode of choice. Other applications might include cocarcinogenesis testing where a defined concentration of a known carcinogen is to be applied along with emissions test material. Papers describing the application of this technique are included in the references for this section.

B. Oral

Although the oral route is likely to be only a minor route of human exposure, its possibility of occurrence dictates its inclusion in these guidelines. This route has been employed for the bulk of systemic toxicological studies for many years.

For administering test materials via the oral route two modes may be employed: (1) incorporation into food or water; and (2) gavage (stomach tube). Although (1) may be easier and simpler in many cases, gavage offers closer control of dose of exposure agent, less wastage of test materials, and may be the preferred mode of oral exposure in many experimental situations.

C. Eye and Skin

For motor vehicle emissions, eye and skin contact are important routes of human exposure. The health effects of exposure via the eye and skin routes are generally considered to be primarily transient irritation; however, the eye possesses a potentially high sensitivity to toxic substances and should receive careful attention as a potential target organ for fuels and fuel additives both before and after combustion. For example, a splash of unburned test material or simply exposure to vapors of test material may result in dulling of the cornea and loss of epithelial cells - symptoms of exposure to organic solvents.

IV. References for Route and Mode of Exposure

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V. HEALTH RELATED TESTS

A. Toxicity

1. General
    - a. Oral
        - (1) LD_{50} Determination
        - (2) Blood/Urine Analysis
        - (3) Gross and Histopathology
    - b. Dermal
    - c. Eye
    - d. *In Vitro* Toxicity Tests
        - (1) Tissue Slices
        - (2) Primary Cells
        - (3) Cell Lines
2. Pulmonary
    - a. LC_{50} Determination
    - b. Host Defense Mechanisms
        - (1) Infectivity Model System
        - (2) Mucociliary Function
            - Ciliary Activity
            - Mucociliary Transport
            - Mucus Production and Characteristics
        - (3) Free Lung Cell Populations
        - (4) Alveolar Macrophage Function
            - *In Vivo* Exposure
            - *In Vitro* Exposure
        - (5) Immune Function

   c. Pulmonary Function
   d. Gross and Histopathology
   e. Blood/Urine Analysis
3. Central Nervous System/Behavior

V. HEALTH RELATED TESTS

A. Toxicity

1. General

a. Oral

(1) LD_{50} Determination

Acute toxicity tests are designed to determine the harmful effects of an agent resulting from exposure to a single dose. Selected effects (e.g., altered physiological, anatomical, or behavioral parameters, or death) are monitored for a specified period of time. The most commonly used criterion for such a test is the LD_{50} (the dose which results in death of half of the exposed animals) with its associated confidence limits. In order to most efficiently determine this value, without the requirement to use excessively large numbers of experimental animal groups, an initial range-finding study is performed. Small groups of animals, usually two per group, are administered the test agent, covering a wide concentration range. It is generally recommended that the dosage steps increase by a factor of 2 and cover a sufficiently wide range so that a no-effect as well as a consistently lethal dosage can be determined (Loomis, 1974).

For determination of acute toxicity by the oral ingestion route or by skin penetration, standard procedures used for general toxicological testing should be appropriate.

The LD_{50} test determines the dosage of test material which results in death for 50 percent of the exposed animals in a specified time period. Test animals, usually rats, are exposed to graded dosages of the test material, the range of which has been previously determined in a range-finding experiment. Dosage intervals are generally chosen for mathematical or statistical convenience, i.e., increasing the dosage by a factor of 1.26 or the equivalent of 0.1 log interval, from which it is then simple to compute the LD_{50} from published formulas and tables.

The number of animals to be used at each dosage level should be considered in terms of the type of information desired from the test, i.e., statistical validity and slope and shape of the dose-response line. Generally a minimum of 10 animals should be assigned to each group, either of the same sex or an equal number of each sex (EPA, Pesticides Guidelines, 1977).

In order to obtain the maximum amount of information from the acute toxicity test which will be of value in designing subsequent sub-acute testing, extensive evaluation of all harmful effects should be performed. In addition to the obvious criterion of lethality, monitoring of a variety of behavioral and physiological symptoms such as described by Loomis (1974) would be appropriate.

The choice of animal species is generally based on previous work in the area; thus, the most commonly used are rats and mice. However, species variability in response should be considered on the basis of known toxicity in various species of similar or related compounds to the one being tested, and the choice of test animal be made accordingly. Similarly, the length of the observation period should be chosen based on best-available information on the time response of similar or related materials. A 24-hour observation period is sometimes sufficient; more commonly, 14 days is employed. In any event, details of experimental materials and methods should be clearly specified in the reporting of results.

V. A. 1. a. References for Oral

(1) LD_{50} Determination

Committee on Toxicology, Arnold J. Lehman, Chairman. (1964) Principals and procedures for evaluating the toxicity of household substances. NAS-NRC Publ. No. 1138, Washington, D. C.

EPA. (1977) Pesticides Guidelines Draft.

Litchfield, J. T., Jr., and Wilcoxon, F. (1949) A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96:99-113.

Loomis, T. A. (1974) Essentials of toxicology, 2nd ed. Lea and Febiger, Philadelphia, 223 pp.

Weil, C. S. (1952) Tables for convenient calculation of median-effective dose (LD_{50} or ED_{50}) and instructions in their use. Biometrics 8:249-263.

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Weil, C. S. and Wright, G. J. (1976) Intra- and interlaboratory comparative evaluation of a single oral test. Toxicol. Appl. Pharmacol. 11:378-388.

(2) Blood/Urine Analysis

For all exposure modes in which systemic toxic effects may be expected, i.e., dermal, oral, and inhalation, alterations in metabolism or physiology may occur. Such metabolic or physiological changes, as well as direct cellular injury, may result in variations from normal patterns of the blood and urine constituents. Consequently, hematology, blood chemistry, and urinalysis are basic parts of routine toxicological testing. Testing should include but not be limited to the following:

Hematology - hematocrit, hemoglobin, erythrocyte count, total and differential leukocyte counts, and reticulocyte count.

Blood Chemistry - calcium, sodium, potassium, chloride, total serum protein, serum protein electrophoresis, serum bilirubin, blood urea nitrogen, fasting blood sugar, and serum enzymes.

Urinalysis - pH, specific gravity, total protein, glucose, ketones, bilirubin, and microscopic examination of sediment.

V. A. 1. a. (cont.)

(2) Blood/Urine Analysis

Committee on Toxicology, Arnold J. Lehman, Chairman. (1964) Principles and procedures for evaluating the toxicity of household substances. NAS-NRC Publ. No. 1138, Washington, D. C.

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Loomis, T. A. (1974) Essentials of toxicology, 2nd ed. Lea and Febiger, Philadelphia, 223 pp.

(3) Gross and Histopathology

Gross necropsy should be performed on all animals which die during the course of toxicology testing by all administration routes; and on all animals at the termination of testing. Gross necropsy reveals evidence of overt injury or disease processes in organ systems, and allows sampling of suspect tissues for subsequent histopathologic examination. In all cases, a complete dissection and necropsy should be performed, and should include examination of the external body surface, orifices, and all thoracic and abdominal viscera.

Suspect tissues observed during the gross necropsy should be prepared for histopathologic examination, following suitable fixation and staining. Additionally, histopathologic examination should form a part of the overall toxicologic profile for all subacute and chronic testing routines, regardless of route of administration. Minimal examination should include: all gross lesions, brain, spinal cord, eye, pituitary, salivary gland, heart, thymus, thyroids, parathyroid, lungs, trachea, esophagus, stomach, small and large intestine, adrenals, pancreas, liver, gall bladder, kidneys, urinary bladder, aorta, testes, prostate, ovaries, corpus and cervix uteri, spleen, at least 2 lymph nodes, bone and marrow, skeletal muscle, skin, sciatic nerve, and mammary gland. Additionally, for inhalation route of exposure, the examination should include particular attention to tissues of the respiratory tract, including the trachea, bronchi, and paranasal sinuses.

Variations in requirements for histopathologic examination of animals in different dosage groups are detailed elsewhere (Pesticides Guidelines Draft, 1977) and appear to also be appropriate for toxicity testing of fuels and additives. A valuable guide to the methodology for pathological assessment of pulmonary toxicity is also found in the 1976 review paper by Dungworth _et_ _al_.

V. A. 1. a. (cont.)

(3) Gross and Histopathology

Dungworth, D. L., Schwartz, L. W., Tyler, W. S., and Phalen, R. F. (1976) Morphological methods for evaluation of pulmonary toxicity in animals. Annu. Rev. Pharmacol. Toxicol. 16:381-389.

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V. A. 1. b. Dermal

Assays for dermal toxicity must take into consideration several aspects of possible tissue injury resulting from the presence of the test material. These include corrosive action or gross destruction of dermal tissue; primary irritation or a local nonimmunological inflammatory response which is reversible and does not lead to tissue destruction; and contact sensitization, an immunologically mediated reaction resulting generally from repeated exposure and manifested by inflammation, edema, and often formation of blisters at the site of contact. Acute dermal toxicity results from penetration of the skin by the test material, with systemic spread of the material by the circulation resulting in toxicity similar to that caused by other routes of entry such as oral or respiratory.

Assays for primary irritation or corrosion generally use the albino rabbit as the test animal, although other animals, in particular the guinea pig, could be used. An albino animal is preferred in order to aid visualization of responses. Comparison of the rabbit and guinea pig (Roudabush *et al.*, 1965; Nixon *et al.*, 1975) indicates that the latter can reliably serve as a substitute for the rabbit, but there are indications that neither is a consistently reliable indicator of human responses. This is particularly true with respect to comparisons made using abraded skin and it has been suggested (Nixon *et al.*, 1975) that the abraded skin test be omitted, since interpretation of results are made much more difficult when the test materials are applied to abraded skin.

The use of a 4-hour test period rather than the 24-hour period as originally described by Draize (1944) has been suggested as a more pertinent indicator of actual human exposure to many potentially hazardous materials. Critical evaluation of the 4-hour test for skin penetration toxicity (Weil *et al.*, 1971) indicated that the shorter testing period correlated well with the longer period, and that one can be reliably converted to the other using mathematical computations.

Despite numerous examples in the literature, the use of animal models to reliably predict human contact sensitivity to various chemicals is still an inexact science. This is the case largely because of subtle differences in the immune response systems of various animals, including man, reflected in different responses of various species to a similar antigenic challenge. The usual animal species used for determination of potential of a material for contact sensitization is the albino guinea pig, since this species develops excellent delayed type hypersensitivity responses to many antigens. Confirmation of the skin sensitizing potential of materials shown to be positive in the guinea pig is generally made in human volunteers. However, it should be appreciated that sensitization of volunteers may have long-lasting effects, with the result that subsequent contact with the sensitizer years later may produce adverse symptoms.

Methods for the study of acute dermal toxicity, i.e., systemic toxicity arising from penetration through the skin by materials applied topically, follow the general procedures for acute toxicity testing by other routes. That is, initial range-finding studies should be conducted in rats or mice, increasing the dosage by either geometric or logarithmic progression. Methods for application of test material to the skin are described by Draize *et al*. (1944), and should be considered in the context of the potential conditions of human exposure to the materials. That is, duration of exposure to the material, potential for normal evaporation from the skin, covering the test site with a semipermeable or impermeable dressing, etc., must be decided on the basis of projected contact with the materials by consumers.

V. A. 1. b. References for Dermal

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Committee on Toxicology, Arnold J. Lehman, Chairman. (1964) Principles and procedures for evaluating the toxicity of household substances. NAS-NRC Publ. No. 1138, Washington, D. C.

Davies, R. E., Harper, K. H., and Kynoch, S. R. (1972) Interspecies variation in dermal reactivity. J. Soc. Cosmet. Chem. 23:371-381.

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Draize, J. H., Woodard, G., and Calvery, H. O. (1944) Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 82:377-390.

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Weil, C. S., Condra, N. I., and Carpenter, C. P. (1971) Correlation of four-hour versus twenty-four hour contact skin penetration toxicity in the rat and rabbit and use of the

V. A. 1. b. (cont.)

former for predictions of relative hazard of pesticide formulations. Toxicol. Appl. Pharmacol. 18:734-742.

Wester, R. C. and Maibach, H. I. (1975) Percutaneous absorption in the rhesus monkey compared to man. Toxicol. Appl. Pharmacol. 32:394-398.

V. A. 1. c. Eye

This is a measure of the potential of a chemical to induce corrosive or irritative changes in a highly sensitive organ (eye) which may also be an important target organ for human exposure. The most commonly used procedure is adapted from the method of Draize _et al_. (1944) and involves the use of rabbits. A measured quantity, usually 0.1 ml of liquids or 10.0 mg of powered insoluble chemicals, is placed in the conjunctival sac of rabbits. The resulting ocular response is determined by visual observation at 24, 48, and 72 hours. Responses are graded according to a carefully defined grading system based on damage to the various ocular structures and duration of injury. A similar evaluation of eye damage could also be performed following exposure of animals to aerosolized test materials.

The use of rabbits as test animals has been the subject of some criticism, based generally on the premise that the eye of the rabbit is not sufficiently similar to the human eye to permit completely reliable extrapolation. A comparative study, using the rhesus monkey and the rabbit and comparing the results to human volunteers (Beckley _et al_., 1969), confirmed the utility of the monkey in predicting human response. However, cost, availability, and other factors may preclude widespread use of nonhuman primates for such testing. On the other hand, the use of smaller species such as guinea pigs and rats may not be appropriate because of constraints imposed by the small size of the eye. In the rabbit, the relatively large size of the eye facilitates instillation of test materials and subsequent observation for tissue injury.

V. A. 1. c. References for Eye

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Committee on Toxicology, Arnold J. Lehman, Chairman. (1964) Principles and procedures for evaluating the toxicity of household substances. NAS-NRC Publ. No. 1138, Washington, D. C.

Draize, J. H., Woodard, G., and Calvery, H. O. (1944) Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 82:377-390.

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V. A. 1. d. In Vitro Toxicity Tests

In vitro toxicity tests can be performed with a large variety of freshly isolated and/or continuously proliferating mammalian cell systems. The types of toxicity tests can be divided into four general categories, including a) cell growth inhibition tests, b) cell survival studies, c) biochemical toxicity tests, and d) functional toxicity tests. Individual assays from each of the above categories can be applied to any given cell system; the selection of cell system should be based on its relationship to species (and metabolic capabilities of the cells of that species), tissue of origin (as close as possible to the expected in vitro route of exposure, e.g., lung cells), and desired endpoint.

Cell growth inhibition tests include effects of continuous exposure of chemicals on the increase in concentration (cell/ml) of cells in exponential growth after a defined period of time (i.e., 48 or 72 hr), or on the accumulation of total cell protein per container (e.g., flask, dish, or tube) of cells in exponential growth.

Cell survival studies include measurement of the cloning efficiency of cells which attach to solid substrates (e.g., glass or plastic) or measurement of the ability of the cells to form colonies in soft agar. The cells can be treated prior to the assay for both of the above, or during the assay for surface attached cell lines. Dye exclusion procedures can be employed for nonproliferating cell lines, although consideration must be given to possible nonlethal membrane effects by the chemical on the measurement.

Biochemical toxicity tests include measurement of DNA precursor incorporation (DNA synthesis) during a continuous treatment interval or for a short period of time (rate study) immediately after a treatment period; RNA precursor incorporation (RNA synthesis) for a short period of time (rate study) immediately after a treatment period; or amino acid incorporation into protein for a short period (rate study) immediately after a treatment period.

Functional toxicity tests are relevant to the specific cell system selected, e.g., the alveolar macrophage, as described below.

(1) Tissue Slices

Some of the biochemical and functional toxicity tests mentioned above could be performed on slices of appropriate tissues. An example is the assay of effects of in vitro exposure upon ciliary beating employing slices of tracheal tissue. (See Pulmonary Toxicity, V. A. 2. b. (2)).

(2) Primary Cells

Primary cells may be utilized for *in vitro* determination of functional and biochemical toxicity effects. An example is the *in vitro* alveolar macrophage test system described under Pulmonary Host Defense Mechanisms (See V. A. 2. b. (4)).

(3) Cell Lines

Continuous mammalian cell lines could be used for all four categories of *in vitro* toxicity tests described above. The cell lines chosen should include a normal human diploid cell line for reference purposes, but should also include a cell system appropriate to the endpoint being measured or to previous standardization experience. Consideration should be given to use of the cell lines employed by the *in vitro* screening program of the National Cancer Institute for cell growth inhibition studies and to the use of the same mammalian cell lines recommended for *in vitro* chemical transformation and mutagenesis for the cell survival studies. These same cell systems can of course be employed for biochemical toxicity tests, provided they contain the appropriate enzymes necessary for metabolic activation of potentially hazardous chemicals.

V. A. 1. d. References for In Vitro Toxicity Tests

(1) Tissue Slices

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Sayeed, M. M., Senior, R. M., Chaudry, I. H., and Baue, A. E. (1975) Characteristics of sodium and potassium transport in the lung. Am. J. Physiol. 229:1073-1079.

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Yeager, H., Jr., and Massaro, D. (1972) Glucose metabolism and glycoportein synthesis by lung slices. J. Appl. Physiol. 32: 477-482.

V. A. 1. d. (cont.)

(2) Primary cells

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V. A. 1. d. (cont.)

(3) Cell Lines

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Eagle, H. and Foley, G. E. (1958) Cytotoxicity in human cell cultures as a primary screen for the detection of anti-tumor agents. Cancer Res. 18:1017-1025.

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V. A. 2. Pulmonary

a. LC_{50} Determination

Acute inhalation toxicity tests generally involve determination of the LC_{50} and gross pathologic examination of animals killed by exposure. Standard, routinely used test procedures should suffice for obtaining this information for the uncombusted materials, with particular attention being given to choice of solvents, solvent controls, etc. In the case of exhaust products, difficulties may be encountered in obtaining sufficiently high concentrations to determine lethal toxicity of specific exhaust components due to the dilution necessary to reduce carbon monoxide content to nonlethal levels. The usual experimental animal for such studies is the rat; exposure is usually for a minimum of 1 hour, followed by daily observation for adverse symptoms and recording of mortality for a subsequent 14-day period. Dead animals should be subjected to complete gross pathologic examination, examining all appropriate organ systems. At the end of the test period, all surviving test animals are similarly subjected to complete gross pathologic examination. These tests provide information on the relative potential of the test material to cause irreversible alterations in various organ systems.

V. A. 2. a. References for Pulmonary LC_{50} Determinations

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V. A. 2. a. (cont.)

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V. A. 2. b. Host Defense Mechanisms

(1) Infectivity Model System

Sterility of the lower airways is maintained through the cooperative interactions of a complex system comprising both mechanical and biological components. It has been shown that increased susceptibility to respiratory infection results from inhalation of pollutants capable of compromising the functional integrity of any of the individual elements of the pulmonary defense system. The increase in susceptibility may be effected by activation of latent infections once surveillance and defense mechanisms are compromised, or by the failure of host defense systems to respond adequately to subsequent bacterial invasion. The consequences of the decrease in resistance to respiratory infection can be life-threatening for certain populations such as the elderly.

Whether or not a specific pollutant atmosphere has a detrimental effect upon the respiratory defense system can be determined by small animal screening studies (Gardner et al., 1977). For the present situation of exposure of human populations to motor vehicle emissions of varying composition, this test offers a valuable means of detecting and comparing direct effects of inhaled test materials. Endpoints measured are usually alterations in mortality or survival time of animals exposed to aerosols of viable bacteria.

If an alteration in resistance to infection is observed, specific elements of the overall defense system can then be studied to allow for identification of high-risk populations and possible protective or therapeutic measures. Separable elements of the respiratory defense system which can be studied include: alveolar macrophage function, mucociliary transport, ciliary beating, and mucus physical properties. In performing these tests, exposure to the bacteria may either precede or follow exposure to the test material, depending upon the nature of the experimental design. Particular attention should be given to several details of the exposure protocol: selection of the infectious agent should be made after consideration of consistency and reproducibility of infective dose, lethality for the particular test animals being used, logistics of handling and aerosolizing infective agents which may be of human health significance, etc. The uniformity of bacterial exposures for all animals in a group should be confirmed by determining bacterial loading of animals placed at different positions in the exposure chamber. Selection of the length of observation time should take into consideration the particular characteristics of both the test animal and the infectious agent being used. An observation time of 14 days is commonly used, during which time the number of animals surviving at each day of observation is determined and the mean survival time calculated by standard formulas.

Bacterial inactivation and killing, and rates of clearance, can be ascertained by using radiolabeled bacteria. Following exposure of the animals to the labeled agents, total bacterial dose can be determined by homogenizing the lungs of exposed animals, determining the amount of radioactive label present, and extrapolating the number of bacteria from a predetermined standard, relating amount of label with numbers of bacteria. At various time periods after exposure to the test material, and following exposure to the labeled infectious agent, clearance of bacteria from the lung can be similarly determined, as well as the efficiency of killing of the bacteria by macrophages or other defense mechanisms. For determination of inactivation or killing efficiency, lungs are removed from infected animals at various time intervals after exposure, the amount of radioactivity (thus the total number of bacteria present) is determined, and the number of viable bacteria determined by standard dilution and culture techniques (Goldstein _et al_., 1976).

(2) Mucociliary Function

Mucociliary function constitutes one of the three primary defense systems of the respiratory tract, and may be the most important of the three with respect to removal of inhaled bacteria deposited in the conducting airways. It is a cooperative process involving both the beating of cilia attached to ciliated epithelial cells and the consequent upward motion of the overlying mucus blanket. The system may be thought of as a continuously moving mucus escalator transporting particles and cells out of the lung and eventually into the gastrointestinal tract. For proper function, this system depends upon a constant supply of mucus of the right viscosity. Therefore, if either the beating of the cilia or the supply of mucus of the right viscosity is disturbed, clearance of particulates from the respiratory tract is impaired.

The separate mechanisms involved in mucociliary function are all extremely vulnerable to inhaled pollutants. Ciliary beating has been shown to be inactivated by cigarette smoke and mucociliary transport is inhibited by a number of substances of particular relevance to fuels and fuel additives such as acrolein, formaldehyde, acetone, cadmium, SO_2, NO_2, NH_3, and cigarette smoke (Goldstein _et al_., 1976; Adalis _et al_., 1977)

Ciliary Activity

Assays of ciliastic activity of various agents are performed _in vitro_ following _in vitro_ or _in vivo_ exposure of the trachea, measuring changes in ciliary rate by either visual or nonvisual methods, and then comparing either changes in beating rate or in time for cessation of ciliary movement for both control and test specimens. Many technical variables are inherent in this methodology, a primary one being observer error in judging ciliary rate or cessation of beating. Because of all the variables, statistical evaluations using blind protocols and randomized testing are requisite to the performance of this assay. The preferred method of measurement of ciliary rates is by means of high-speed photography, or other objective methods using photoelectric or stroboscopic techniques.

Mucociliary Transport

Mucociliary transport rates are determined by visually monitoring the rate of movement of particles placed within an excised tracheobronchial system, or an incised and externalized *in situ* system or by externally monitoring the movement of inhaled radioactive particles in intact animals and humans. Visual methods usually employ carbon particles, india ink, or graphite, the movement of which is observed microscopically within the trachea. The human subject or the experimental animal inhales radioactive particles from an aerosol generator; sequential measurement of particle locations are then made with a gamma-ray-detecting camera, such as an Anger camera.

Mucus Production and Characteristics

Rates of mucus production are measured by collecting secretions from an anesthetized animal via an intratracheal or endotracheal cannula, or by a method which involves isolation of a short segment of cervical trachea of a dog and formation of the segment into a subcutaneous pouch.

Increases in either viscosity or elasticity of mucus are associated with reductions in mucociliary function, therefore measurement of visco-elastic properties of mucus as a function of exposure to a given agent provide valuable information. The physicochemical properties of mucus are due to large molecular weight aggregates of several distinct glycoproteins which are in random coil configuration in the aqueous solution. Irritant, water soluble gases such as SO_2 have been shown to increase mucus viscosity (Litt *et al*., 1976). An increase in mucus viscosity causes a slowing of mucus flow resulting in abnormal retention and accumulation of particulates.

(3) Free Lung Cell Populations

Following inhalation exposure, alterations in numbers and types of free lung cells could provide information useful in predicting possible impairment in host defense against inhaled infectious agents, or inflammatory or other tissue injury. Free cells generally are comprised of predominantly alveolar macrophages, with small percentages of lymphocytes, polymorphonuclear leukocytes, and occasionally other cell types. The numbers and distribution of these cell types can be significantly altered following insults of various kinds; induction of inflammatory changes may lead to marked increases in polymorphonuclear leukocytes and macrophages, antigenic stimulation may lead to increased lymphocyte numbers, particulate stimulation may lead to increased numbers of alveolar macrophages, etc. (Goldstein *et al*., 1976).

For determination of numbers and distribution of lung cells, procedures are available for routine lavage of the lungs of various experimental animals. Particular attention should be given to the composition of the lavage fluid; more complete recovery of free cells is accomplished with the use of calcium and magnesium free solution. Cells thus recovered can be washed, counted by standard laboratory procedures (hemacytometer or particle counter), and viability determined by ability to exclude vital dyes. Differential determinations should be made following preparation of smears on microscope slides and staining with standard hematological techniques (Brain, 1970).

(4) Alveolar Macrophage Function

In Vivo Exposure

Alveolar macrophages function as primary effectors of host defense against inhaled particulate matter such as bacteria, viruses, and fungi, which are ingested, killed, and degraded by the macrophages. Nonbiological particles such as dusts, soot, etc., are also ingested and degraded to varying extents, unless their chemical composition makes them cytotoxic to macrophages, in which case the particles are released from dead cells to be taken up by other macrophages and repeat the cycle. The separate stages of macrophage function can be inhibited individually - i.e., metabolic inhibitors could preferentially inhibit intracellular bacterial killing without affecting phagocytosis, or could cause a delayed response such as labilization of lysosomal membranes leading to cell injury or death without affecting phagocytosis rates or numbers of particles taken up before sufficient quantities of hydrolytic enzymes had leaked into the cytoplasm to injure the cell. However, phagocytosis measurements are easily and readily performed, are useful for comparative toxicity studies, and, if performed in conjunction with a careful quantitative estimation of cell viability, will provide an assessment of alterations in macrophage function.

Alterations in function of alveolar macrophages subsequent to inhalation of test materials can be estimated on the basis of ability of cells recovered from animals subsequent to exposure to exclude dyes and capacity of the recovered cells for phagocytosis of particles. Phagocytosis is frequently assayed on macrophages which have been allowed to attach to a glass or plastic surface, with the cells which did not attach within a given period of time removed by decantation. The attachment process, however, may represent a process of selection for metabolically activated cells (i.e., those with a high energy charge) over those in a resting, metabolically depleted, or immature stage, and since it is presently not clear how this compares with the normal state of macrophages within the lung, consideration should be given to comparison of phagocytic capabilities for both suspended and attached cells.

Phagocytosis can be expressed as numbers of particles ingested per cell and numbers of cells taking up particles, both of which measurements have relevance to assessment of macrophage function.

Two methods of estimating impairment of endocytosis which may provide an index of cytotoxicity of particulate material or of other substances inhaled concurrently should be of particular value for fuels and fuel additives (Brain and Corkery, 1977; Graham *et al*., 1975).

Another aspect of macrophage function which is likely to be altered by inhalation of test emissions is the activity of lysosomal enzymes. The enzymes of the lysosomes represent the means by which substances taken in by phagocytosis are degraded; or by which physiological protein, lipid and carbohydrate molecules are broken down. Extracellular release of lysosomal proteases as a consequence of phagocytosis of particulates may produce emphysema-like lesions and may be the initiating factor in emphysema. Inhalation of test emissions could cause an increase in release of lysosomal enzymes either by cytotoxicity to macrophages resulting in cell lysis, or by the increased phagocytic load (Rogers *et al*., 1976; Janoff *et al*., 1971; Traurig, 1976).

In Vitro Exposure

Alveolar macrophage function consequent to *in vivo* exposure to pollutant atmospheres frequently provides a sensitive index of pollutant effect. The capacity of a specific pollutant for affecting lung macrophage function can also be screened by exposing isolated macrophages *in vitro*. Specific parameters which are measured in the macrophage test system of Waters *et al*. (1975) include: phagocytosis, viability, cell number, ATP concentration, depression of respiration, and lysosomal enzyme activities.

(5) Immune Function

The possibility that exposure to various chemicals may lead to compromised immunological capabilities is currently being recognized as an important aspect of toxicological assessment of hazard. Of particular importance are toxic effects which may be manifested as development of various degrees of immunosuppression, either by virtue of interference with immunologic recognition processes or by indirect effects on the cell populations necessary for normal immunological function. In order to adequately examine the effects of such agents on immune systems, it is necessary to be able to detect alterations in both compartments of the immune response, i.e., humoral and cell mediated. A variety of assay systems are available for studying effects of perturbing agents on both of these systems.

The usual procedure for assessing changes in humoral or antibody mediated immunity involves immunization of a suitable test animal, frequently mice or rabbits, with heterologous erythrocytes or another suitable antigen and subsequently measuring antibody titers, or quantitating numbers of antibody producing spleen cells by a plaque assay. The latter has the advantages of permitting determination of both IgG and IgM producing cells, important parameters which may be related to susceptibility to infection or development of other disease processes; it also is generally done with mice, thus minimizing the cost of the assay.

Evaluation of effects on the cellular immune system may take many forms. These may include studies of hematological and histopathological alterations in the processes responsible for maintenance of effective cell populations in various organs including blood and lymph nodes; direct measurements of functional integrity of immunologically relevant cells including lymphocytes and macrophages; or endpoint measurements of response to immunization with antigens known to evoke responses in the cell mediated compartment of the immune system, e.g., skin sensitization to tuberculin. Under conditions of suspected high risk of interference with the immune system, it would be valuable to perform assessment of lymphocyte and macrophage function, using such test procedures as: blastogenic responsiveness of lymphocytes to both T and B cell mitogens and defined antigens to which the animals have been sensitized; capability of lymphocytes to produce and release selected mediators of cellular immunity such as macrophage migration inhibitory factor or lymphotoxin; and assessment of the capability of macrophages from test animals to function normally in processes related to immune responsiveness, i.e., response to migration inhibitory factor, phagocytic capability, and capability to co-operatively interact with lymphocytes.

V. A. 2. b. References for Host Defense Mechanisms

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V. A. 2. b. (cont.)

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V. A. 2. b. (2) (cont.)

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V. A. 2. b. (cont.)

(2) Mucociliary Function

Ciliary Activity

In vivo exposure

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V. A. 2. b. (2) (cont.)

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(4) Alveolar Macrophage Function

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V. A. 2. c. Pulmonary Function

Assessment of pulmonary function following inhalation exposure to fuels and/or fuel additives would provide information about lung damage of a different nature than that derived from morphological studies alone. The complementary information that could be derived from a combination of morphology and lung function, in those experimental animal systems which lend themselves to those types of studies, indicates that both types of studies should be performed; i.e., histopathological studies for overt tissue injury, and lung function studies for detecting alterations which may provide evidence of functional impairment. Measurement of changes in respiratory rate alone is a simpler procedure for which several advantages can be considered: the procedure is sufficiently simple to permit its use in a screening program; the results are highly reproducible and permit formulation of dose-response curves; and it is sufficiently sensitive to permit-detection of irritating effects at very low concentrations of test materials where pathological tissue injury would not be expected to be seen.

Gaseous irritants such as sulfur dioxide, formaldehyde, acrolein, formic acid, and sulfuric acid cause an increase in pulmonary flow resistance, a slight decrease in compliance, and at higher concentrations, a decrease in breathing frequency. Irritants produce inflammation and inflammation of the upper respiratory tract leads to rhinitis, pharyngitis, and laryngitis; inflammation of the bronchi results in bronchitis and bronchopneumonia; while inflammation of the lungs leads to pulmonary edema and pneumonia.

A complete pulmonary function profile would include: volume studies, including total lung capacity and its subdivisions, vital capacity and functional residual capacity - for detection of injury compatible with emphysematous or fibrotic changes; pressure-volume curve (compliance) as a measure of elasticity; airway flow characteristics (conductance) to provide information about flow-volume relationships; closing volume, which determines at what volume the small airways close, thus detecting damage to smaller airways in the basilar portion of the lung; and diffusing capacity which determines the characteristics of gas movement from the alveoli into the blood and thus provides a measure of damage to capillaries, the alveolar units, or both which may result in impaired gas exchange. Along with the pulmonary function studies, additional useful information can be obtained by monitoring the levels of blood gases, including O_2, CO_2, and the blood pH.

Determination of whether or not to require pulmonary function studies would have to be made after consideration of such factors as cost and applicability of the procedures for the particular animal models selected. A complete workup is difficult or impossible to perform with small animals such as rats or hamsters; selected types of information can be obtained, however. For larger animals, such as dogs or subhuman primates, complete pulmonary function studies could be done, and could provide valuable information with significant clinical relevance.

V. A. 2. c. References for Pulmonary Function

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V. A. 2. d. Gross and Histopathology

Refer to V. A. 1. a. (3).

V. A. 2. e. Blood/Urine Analysis

Refer to V. A. 1. a. (2).

V. A. 3. Central Nervous System/Behavior

Behavioral toxicology as a sensitive indicator of neurotoxicity has developed rapidly within the past decade and is expected to become an increasingly important arm of environmental toxicology as its development continues. It is recognized that neurotoxicants introduced into the environment affect the central nervous system (CNS) through a variety of mechanisms and that these CNS effects are manifested as modifications in behavior. The question now arises as to what extent behavioral problems confronting society may be due to actions of environmental contaminants. For example, some childhood behavioral disorders (hyperkinesis, minimal brain dysfunction, etc.) appear to be of epidemic character and could be due to chemical factors in the environment; both lead and food additives have been proposed as causative factors. Neurotoxicity is recognized to be manifested as behavioral modification related to severity of dose of exposure to heavy metals such as manganese, lead, and mercury; organophosphate pesticides; organic solvents; and carbon monoxide. At another level, more subtle psychological affects appear to be related to environmental chemical exposure. Occupational exposure to organophosphate pesticides has been linked to higher anxiety levels (Levin *et al*., 1975), and carbon disulfide exposure, which produces a broad range of behavioral and neurological disorders, has been associated with increased risk of death from coronary heart disease and suicide.

The susceptibility of the CNS to chemical toxicants is enhanced in the immature and aged individual, and the CNS may be particularly vulnerable in the fetal stage. In fact, behavioral teratology may prove to be uniquely significant in the detection of: (1) reproductive effects as a consequence of continuous, environmental exposure of both parents and of the offspring pre- and postnatally; and (2) mutagenic processes may first be detected as expressions of behavior (DHEW-NIEHS, 1977).

In considering the potential health effects of fuel and fuel additive exposures, it should be remembered that different neurotoxicants whose behavioral expressions are mediated through a common mechanism could result in additive effects which would have to be taken into account in the regulation of environmental levels.

The assessment of neurotoxicity of a given agent can be approached in either of 2 ways: (1) through a sequential series of tests of increasingly specific nature (Weiss *et al.*, 1975); and (2) by a single sensitive, complex performance task. The sequential approach is economically attractive for screening of potentially neurotoxic agents; however, a compound with only subtle effects would require a long series of tests with essentially negative findings in this approach. On the other hand, more complex behaviors and performances are more sensitive to brain lesions and drugs than simpler behaviors and performances. Therefore, if the most sensitive assay for detection of the lowest level of exposure of environmental contaminant is sought, complex performance measurement may be the most meaningful (DHEW-NIEHS, 1977). Disadvantages of the complex performance approach are the inherent time and cost involved.

If a neurotoxicant interacts with the CNS strongly enough, gross behavioral changes can be detected in small animals as convulsions, ataxia, and the loss of various reflexes. These gross behavioral changes may be employed in acute, range-finding experiments for at least some forms of fuel and fuel additive test materials; additive ingredients, technical grade materials, and use-formulations could be screened by such measurements. These initial screening measurements include: (1) activity changes - i.e., motor functions; (2) objective signs such as tremors, ptosis, salivation, lacrimation, defecation, convulsions, abnormal postural changes, etc.; (3) reflex changes - hyper- or hyporeflexia; (4) elicited responses - grasping, response to pinch, induced convulsions, orientation, etc.; and (5) body weight changes, immediate and brief weight loss and failure to gain normally. These tests and other appropriate tests are used by the pharmaceutical industry as initial screens for CNS activity and could be of significant predictive value for fuel and fuel additive testing (NAS-NRC, 1975). If these initial screening tests show CNS activity they should be followed by more definitive behavioral toxicology tests as described above.

V. A. 3. References for Central Nervous System/Behavior

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V. B. *Mutagenesis*

- 1. Gene Mutations
  - a. Procaryotic Microorganisms (Bacteria)
    - (1) *Salmonella typhimurium*
    - (2) *Escherichia coli* WP2
    - (3) *Escherichia coli* K12
  - b. Eukaryotic Microorganisms
    - (1) *Neurospora crassa*
    - (2) Haploid Strains of Yeast
  - c. Plants
    - (1) *Tradescantia*
  - d. Insect
    - (1) *Drosophila melanogaster*
  - e. Mammalian Cells
    - (1) Mouse Lymphoma, L5178Y
    - (2) Chinese Hamster Lung, V79
    - (3) Chinese Hamster Ovary, CHO
  - f. Rodent
    - (1) Mouse Specific Locus
    - (2) Mouse Isozyme Specific Locus
- 2. DNA Damage and Repair
  - a. Repair-defective Microorganisms
    - (1) Recombinationless Mutants of Bacteria
    - (2) DNA Polymerase-deficient Mutants of Bacteria
    - (3) Normal versus Repair-deficient Yeast
  - b. Mammalian Cells
    - (1) DNA Single-strand Breaks
    - (2) Unscheduled (Nonreplicative) DNA Synthesis

- c. Rodent Germinal Cells

3. Chromosomal Effects
    - a. Yeast (Mitotic Recombination)
    - b. Insect (Drosophila)
        - (1) Dominant Lethality
        - (2) Chromosomal Rearrangements
    - c. Mammalian Cells
        - (1) Chromosomal Aberrations
        - (2) Sister Chromatid Exchange
    - d. Rodents
        - (1) Cytogenetic Analysis
            - Bone Marrow
            - Circulating Peripheral Lymphocytes
            - Spermatocytes
        - (2) Heritable Chromosomal Damage
            - Sex Chromosome Loss
            - Dominant Lethal Effects
            - Heritable Translocation Test

4. Methodological Considerations
    - a. Metabolic Activation
        - (1) *In vivo*
        - (2) *In vitro*
    - b. Treatment Conditions

V. B. Mutagenesis

1. Gene Mutations

Induced gene mutations include dominant gene mutations and recessive gene mutations. The molecular processes responsible for either can be point mutations, including base-pair substitutions, additions, or deletions, or small interstitial deletions, defined as chromosomal deletions which are too small to be detected microscopically, but which may involve several genes or part of one gene. The latter is likely to result in limited expression, even if recessive, while point mutations are rarely expressed because most are recessive.

The number of genes (markers) in mammals currently available for study are limited; the detection of either dominant or recessive gene mutations in whole animals can require tens of thousands of animals. This limits the use of whole animals for this type of mutagenicity testing, which can be performed with lower organisms.

Induced gene mutations are detected as changes of specific loci or as recessive lethals. Both tests can measure mutations other than gene mutations. The former is measured using forward or reverse mutation, while the latter is usually detected as sex-linked lethals.

In application of any in vitro mutagenesis system, careful attention should be given to the metabolic activation capability of the system; and, if not inherently present in the specific organism, an appropriate metabolic activation system must be employed.

Reverse mutation assays can be performed with a number of unicellular organisms. A major consideration in performing reverse mutation assays is that a given chemical may cause mutation by only one specific molecular mechanism, e.g., base-pair substitution as compared to frameshift mutation, and if the indicator organism only demonstrates reversion due to one of these mechanisms, potentially mutagenic agents can go undetected. To eliminate this difficulty, each system employed should allow for testing against different strains of the same organism which respond to chemicals acting by different mechanisms. In each case, these strains chosen must be of documented genetic character, and a data base must exist for its response to known chemicals acting by different mechanisms.

Forward mutations can be detected in several unicellular biological organisms; they offer the advantage, as compared to reversion mutations, of being caused by several different mutagenic mechanisms. This may be of advantage in determining relative mutagenic potential of a series of chemicals, at least in that organism; this relative ranking would be very difficult when a series of different tester strains are employed for reversion mutation assay. Bacterial systems for detection of forward mutation are being developed.

a. Procaryotic Microorganisms (Bacteria)

Reversion at different loci (e.g., histidine, tryptophan, and arginine loci) in different strains of *Salmonella typhimurium*, *Escherichia coli*, and *Bacillus subtilis*.

The currently available bacterial reverse mutation tests include the histidine reversion test in *Salmonella typhimurium* with associated metabolic activation system (Ames *et al*., 1973, 1975; McCann *et al*., 1975; McCann and Ames, 1976; Frantz and Malling, 1975); reversion tests in *Escherichia coli* K-12, e.g., at the arginine locus (Mohn *et al*., 1974); reversion in *Escherichia coli* WP2 at the tryptophan locus (Bridges *et al*., 1967; Bridges, 1972; Mitchell, 1974; Green and Muriel, 1976). At the present time, the most extensive validation, i.e., positive correlation with known carcinogens and noncarcinogens, has been performed with the *S. typhimurium* histidine reversion assay and reversion in *Bacillus subtillis* 168 I/V at leucine and valine loci (Popper *et al*., 1973).

The immediate advantage of the bacterial level reversion assays is the low cost and rapidity of obtaining test results which can indicate the potential of a chemical to cause mutations. This is not, of course, to say that the chemical will be mutagenic in man, but rather that it has the potential for being so. Presuming appropriate selection of bacterial tester strain, and *in vitro* metabolic activation systems, consideration should also be given to whether the system can respond to insoluble components of the test material (if a mixture), introduction of turbidity into the test plates, and evaporation of volatile agents. The assay will not indicate what effect different routes of entry have on *in vivo* mutagenesis.

In performing reversion assays the tester should use a standardized and documented procedure, maintaining regular checks of the genetic nature of the tester strains. Any modifications of the procedure in order to take into account solubility or volatility of the test agent should be specified, with listing of the criteria for its selection; this is particularly important in view of the possibility that directly or potentially biohazardous components of complex mixtures may be present in small proportions.

Positive controls should be run with any metabolic activation system, and, also, known chemicals (positive controls) must be run in the presence of the test chemical (or mixture) to verify that the latter is not inactivating the metabolic activation system.

The advantage of reverse mutation is its convenience, i.e., mutants can be selected. The advantage over mitotic gene conversion tests in yeast is that the events studied are known to be mutations.

Available bacterial reverse mutation tests include:

(1) Salmonella typhimurium with Mammalian Metabolic Activating System - Assay for Histidine Reversion

The plate incorporation form of this assay employing the improved tester strains is the method which has been employed for validation (Ames et al., 1975). Several modifications of this assay are particularly relevant to the screening of fuel and fuel additive test materials:

Modification of Assay for Volatile Compounds
(Rannug et al., 1974; Bartsch et al., 1975; Ames et al., 1975)

Modification of Assay for Quantitation of Toxicity and Mutagenicity by Use of Liquid Suspension
(Malling, 1971; Ames et al., 1975)

Modification of Assay for Screening of Mammalian Urinary Metabolites
(Durston and Ames, 1974; Commoner et al., 1974)

As pointed out elsewhere in this document, by far the most extensively and rigorously validated of all in vitro mutagen-detection methods is the Salmonella typhimurium standard plate assay of Ames et al. (1975), which incorporates mammalian metabolic activation. The basis for this validation is the screening of some 300 carcinogenic (DNA-reactive) and noncarcinogenic chemical compounds of various structural types, resulting in the detection of approximately 90% of known carcinogens as positive mutagens by the assay (McCann et al., 1975; McCann and Ames, 1976). Further analysis and interpretation of this data has been presented by Bridges (1976); comparisons of the Ames assay with two other bacterial reversion assays which provide additional or complementary information with respect to validation have also been published (Shirasu et al., 1976; Rosenkranz et al., 1976; Bridges, 1976).

The S. typhimurium assay is rapid, requiring 2-3 days for the standard plate assay; inexpensive in terms of both initial investment and operating costs (Drake et al., 1975); reproducible; and provides dose-response data for mutagens with a wide range of mutagenic potency (Ames, 1976). Of particular relevance to the testing of the mutagenic capacity of fuel and fuel additive test materials, this assay has been employed for mixtures (Kier *et al*., 1974; Ames, Kammen and Yamasaki, 1975), and for air-borne polycyclic aromatic hydrocarbon particulates (Tokiwa *et al*., 1977).

The usual methodology of these assays, when a metabolic activation system is employed, is to combine the latter in the agar with the bacteria during the entire incubation period. As indicated in several of the above references, and also by Bartsch *et al*. (1976) and Herbold *et al*. (1977), this "standard" plate assay has many problems, and a suspension treatment prior to incorporation into agar probably should be validated as the "standard" assay (Bartsch *et al*., 1976; Sugimura *et al*., 1976). Other recent developments in bacterial mutagenesis include application of a fluctuation test (Greene *et al*., 1977) and use of concentration gradient plates (Cline and McMahon, 1977).

(2) *Escherichia coli* WP2 with Mammalian Metabolic Activating Systems - Assay for Tryptophan Reversion

(Bridges *et al*., 1967; Bridges, 1972; Mitchell, 1974; Greene and Muriel, 1976)

WP2 is a tryptophan-requiring strain of *E. coli* which can be caused to revert to tryptophan-independence by base substitution; a number of derivatives of this strain are available which are deficient in different DNA repair mechanisms. These repair-deficient strains offer the advantages of: (1) detection of lower doses of mutagen for those strains in which the missing repair process would normally remove the damaged section of DNA; and (2) hypersensitivity of agents damaging DNA for those strains for which the repair process is itself involved in the conversion of DNA damage to the final mutated base sequence. An additional advantage is that it is possible to partly characterize the type of DNA damage produced by a given agent from the pattern of response of repair-deficient strains (Green and Muriel, 1976).

A disadvantage of the WP2 system at present is the lack of an extensive data base required for validation; however, a data base appears to be developing (Shirasu *et al*., 1976; Brusick, 1977).

(3) *Escherichia coli* K12 with Mammalian Metabolic Activating System - Assay for Reverse Mutation at Arginine and Nicotinic Acid Loci and Forward Mutation for Resistance Against 5-methyltryptophan and for Gal$^+$ Phenotype

(Mohn *et al*., 1974; Mohn and Ellenberger, 1977)

K12 (strain 343/113) is a multi-purpose strain of *E. coli* in which both reversion and forward mutations of several genes can be detected. The assay can be performed in spot tests, suspension tests, with mammalian metabolic activation, and in mammalian host-mediated tests (Mohn *et al.*, 1974). In addition to allowing for detection of forward mutation, an advantage of this assay of particular pertinence to the testing of fuels and fuel additives is that it also manifests greater sensitivity toward certain carcinogens, such as nitroheterocylic compounds and dialkylnitrosamines, and it is also more suitable than the *Salmonella* system for host-mediated assay (Sobels, 1977).

As to validation, the data base for this test appears to be severely limited at this time (Brusick, 1977).

V. B. 1. a. References for Gene Mutations - Procaryotic Microorganisms (Bacteria)

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V. B. 1. a. (continued)

(1) *Salmonella typhimurium* Assay

Assay technique/validation:

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V. B. 1. a. (continued)

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V. B. 1. a. (continued)

(2) Escherichia coli WP2/microsome assay

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V. B. 1. a. (continued)

(3) *Escherichia coli* K12/microsome assay

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b. Eukaryotic Microorganisms

(1) Forced Heterokaryons of the Ascomycetes *Neurospora crassa*

The induction of forward mutations in the *ad*-3 locus of a two-compartment heterokaryon of *Neurospora crassa* is considered to be caused by a wide spectrum of genetic events. For the detection of forward mutation due to genetic alterations at the *ad*-3 locus, a very simple procedure involving the appearance of purple colonies is available (de Serres and Malling, 1971). A published data base is apparently limited.

Also employing the two-component heterokaryon of *Neurospora crassa*, it is possible to measure mutation induction resulting from any kind of genetic damage that produces complete inactivation over the entire genome. An estimate of the frequency of recessive lethal damage over the entire genome can be obtained by determining the percentage of heterokaryotic colonies with recessive lethal damage in one of the two components. The recessive lethal mutations in this system result from both point mutations and chromosome deletions (de Serres and Malling, 1971).

(2) Haploid Strains of Yeast

The forward mutation assay in haploid strains of yeast can be performed by detecting loss of function, i.e., certain forward mutations cause resistance to certain anti-metabolites which allow for selective procedures. Alternatively, forward mutation at the so-called red-adenine genes can be determined (Mortimer and Manney, 1971; Brusick and Mayer, 1973; Zimmermann, 1973, 1975).

V. B. 1. b. References for Eukaryotic Microorganisms

(1) *Neurospora crassa*

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(2) Haploid Strains of Yeast

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c. Plants

(1) *Tradescantia* - Pink Mutation of Stamen Hair Cells

Tradescantia clone 02 is a diploid plant (2n = 12), heterozygous for flower color. A change from the normal blue color to pink in cells of stamen hair after chemical treatment is considered a true mutational event because of this heterozygosity. Other types of "aberration events" can also be scored. The exact mechanism of the mutational event causing the change from blue to pink is unknown; it may include deletion events.

The examination of *Tradescantia* stamen hairs for "pink events" as an indicator of mutation appears to be straight-forward, and the system appears uniquely useful for gaseous exposures (Underbrink *et al.*, 1973; Sparrow *et al.*, 1974; Nauman *et al.*, 1976; McNulty *et al.*, 1977). The available data base is limited; this system is still under development.

V. B. 1. c. References for Plants

(1) *Tradescantia*

Underbrink, A. G., Schairer, L. A., and Sparrow, A. H. (1973). *Tradescantia* stamen hairs: A radiobiological test system applicable to chemical mutagenesis. In: Chemical mutagens, Principles and methods for their detection, vol. 3, A. Hollaender, ed., pp 171-207. New York. Plenum.

Sparrow, A. H., Schairer, L. A., and Villalobos-Pietrini, R. (1974). Comparison of somatic mutation rates induced in *Tradescantia* by chemical and physical mutagens. Mutat. Res., 26: 265-276.

Nauman, C. H., Sparrow, A. H., and Schairer, L. A. (1976). Comparative effects of ionizing radiation and two gaseous chemical mutagens on somatic mutation induction in one mutable and two non-mutable clones of *Tradescantia*. Mutat. Res., 38: 53-70.

McNulty, P. J., Nauman, C. H., Sparrow, A. H., Schwemmer, S. S. and Schairer, L. A. (1977). Influence of x-ray dose fractionation on the frequency of somatic mutations induced in *Tradescantia* stamen hairs. Mutat. Res., 44: 235-246.

d. Insect

(1) *Drosophila melanogaster*

The sex-linked recessive lethal test in *Drosophila* is considered applicable to mutation screening since it can detect mutations arising at many loci in the genome, and it can detect the more common types of gene mutations.

In performing the test, genetically appropriate males are treated and mated with genetically appropriate females; daughter females are then individually mated to several of their brothers. The resulting generation is examined in the culture container and the number of cultures which lack normal-eyed (non-Bar) males are an estimate of the number of treated X chromosomes which carry one or more sex-linked recessive lethal mutants. This test system can also be designed to study mutation in treated females (Abrahamson and Lewis, 1971; Vogel and Leigh, 1975). Recently, attention has been given to the capacity of *Drosophila* to metabolically activate pre-carcinogens (Sobels and Vogel, 1976; Baars *et al.*, 1977). Mutations in *Drosophila* have been observed after gaseous exposures (Garrett and Fuerst, 1974); more attention should be given to validation of this system.

V. B. 1. d. References for Gene Mutations - Insect

(1) *Drosophila melanogaster*

Vogel, E. (1976). The relation between mutational pattern and concentration by chemical mutagens in Drosophila. In: Screening tests in chemical carcinogenesis, R. Montesano, H. Bartsch, and L. Tomatis, eds., Lyon, France. IARC Scientific Publications no. 12. International Agency for Research on Cancer.

Vogel, E. and Sobels, F. H. (1976). The function of *Drosophila* in genetic toxicology testing. In: Chemical mutagens, Principles and methods for their detection, vol. 4, A. Hollaender, ed., New York. Plenum.

Abrahamson, S. and Lewis, E. B. (1971). The detection of mutations in *Drosophila melanogaster*. In: Chemical mutagens, Principles and methods for their detection, vol. 2, A. Hollaender, ed., pp 461-487. New York. Plenum.

Vogel, E. and Leigh, B. (1975). Concentration-effect studies with MMS, TEB, 2,4,6-TriCl-PDMT, and DEN on the induction of dominant and recessive lethals, chromosome loss and translocations in *Drosophila* sperm. Mutat. Res., 29: 383-396.

Sobels, F. H.and Vogel, E. (1976). The capacity of *Drosophila* for detecting relevant genetic damage. Mutat. Res., 41: 95-106.

Baars, A. J., Zijlstra, J. A., Vogel, E., and Breimer, D. D. (1977). The Occurrence of cytochrome P-450 and aryl hydrocarbon hydroxylase activity in *Drosophila melanogaster* microsomes, and the importance of this metabolizing capacity for the screening of carcinogenic and mutagenic properties of foreign compounds. Mutat. Res., 44: 257-268.

Garrett, S. and Fuerst, R. (1974). Sex-linked mutations in *Drosophila* after exposure to various mixtures of gas atmospheres. Environ. Res., 7: 286-293.

e. Mammalian Cells

Several mammalian cell culture systems are available for detecting forward mutations. The use of mammalian cell systems is a rapidly developing area, and it will be important to specify choice of mutation system and criteria of selection. The mammalian cell systems are either diploid or aneuploid; they are more complex biochemically and metabolically than the bacterial level mutagenicity systems, which are in several instances "sensitized" to chemical penetration and damage response.

It will be necessary to determine which mammalian cell system is most appropriate for a given test material, taking into consideration the available data base for each system at the time of testing; criteria for selection should be specified. Other important considerations are whether the system selected has the capability of metabolically activating chemicals; and duration of treatment times, since potentially active chemicals may be present in small percentages in a mixture. Currently available mammalian cell assay systems include:

(1) Mouse lymphoma, L5178Y, TK +/- forward mutation at the thymidine kinase locus - assay of Clive and Spector (1975) with and without metabolic activation.

(2) Chinese hamster lung, V79, HGPRT +/- forward mutation at the hypoxanthine-guanine phosphoribosyl-transferase locus (Chu, 1971; Newbold *et al.*, 1977; EPA Pesticide Guidelines, 1977).

(3) Chinese hamster ovary, CHO, HGPRT +/- forward mutation at the hypoxanthine-guanine phosphoribosyl-transferase locus - assay of Hsie *et al.* ~~(1975~~) with and without metabolic activation.

V. B. 1. e. References for Gene Mutations - Mammalian Cells

(1) Mouse lymphoma, L5178Y

Clive, D. Flamm, W. G., and Patterson, J. B. (1973). Specific locus mutational assay systems for mouse lymphoma cells. In: Chemical mutagens, Principles and methods for their detection, vol. 3, A. Hollaender, ed., pp 79-103. New York. Plenum.

Clive, D. (1977). A linear relationship between tumorigenic potency in vivo and mutagenic potency at the heterozygous thymidine kinase (TK +/-) locus of L5178Y mouse lymphoma cells coupled with mammalian metabolism. Abstract no. Bb-3, Presented at Environmental Mutagen Society Meeting, Feb. 1977, Colorado Springs, Colorado.

Clive, D. and Spector, J. F. S. (1975). Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutat. Res. 31: 17-29.

(2) Chinese hamster lung, V79

Huberman, E. and Sachs, L. (1976). Mutability of different genetic loci in mammalian cells by metabolically activated carcinogenic polycyclic hydrocarbons. Proc. Natl. Acad. Sci.,USA, 73: 188-192.

Krahn, D. F. and Heidelburger, C. (1977). Liver homogenate-mediated mutagenesis in Chinese hamster V79 cells by polycyclic aromatic hydrocarbons and aflatoxins. Mutat. Res. 46: 27-44.

Chu, E. H. Y., (1971). Induction and analysis of gene mutations in mammalian cells in culture. In: Chemical mutagens, Principles and methods for their detection, vol. 2, A. Hollaender, ed., pp 411-444. New York. Plenum.

Newbold, R. F., Wigley, C. B. Thompson, M. H., and Brookes, P. (1977). Cell-mediated mutagenesis in cultured chinese hamster cells by carcinogenic polycyclic hydrocarbons: Nature and extent of the associated hydrocarbon-DNA reaction. Mutat. Res., 43: 101-116.

Environmental Protection Agency, (1977). Pesticide guidelines, draft revision.

V. B. 1. e. (continued)

(3) Chinese Hamster Ovary, CHO

Hsie, A. W., Couch, D. B., Brimer, P. A., O'Neill, J.P., and Machanoff, R. (1977). The utility of a quantitative sensitive and versatile mutational assay at the hypoxanthine-guanine phosphoribosyl transferase locus of Chinese hamster ovary cells (CHO/HGPRT assay) in determining the mutagenicity of physical and chemical carcinogens. Abstract to be presented at the American Assoc. of Cancer Research Mtg.

O'Neill, J. P., Brimer, P. A., Machanoff, R., Hirsch, G. P. and Hsie, W. W. (1977). A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system. Submitted to Mutat. Res.

O'Neill, J. P., Couch, D. B., Machanoff, R., San Sebastian, J. R., Brimer, P. A., and Hsie, A. W. (1977). A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Utilization with a variety of mutagenic agents. Submitted to Mutat. Res.

Hsie, A. W., Li, A. P., and Machanoff, R. (1977). A fluence-response study of lethality and mutagenicity of white, black, and blue fluorescent light, sunlamp, and sunlight irradiation in Chinese hamster ovary cells. Mutat. Res., in press.

O'Neill, J. P. and Hsie, A. W. (1977). Chemical mutagenesis of mammalian cells can be quantitated. Nature, in press.

Hsie, A. W., Brimer, P. A., Mitchell, T. J., and Gosslee, D. G. (1975). The dose-response relationship for ethyl methanesulfonate-induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells. Somatic Cell Gen., 1: 247-261.

Hsie, A. W., Brimer, P. A., Mitchell, T. J., and Gosslee, D. G. (1975). The dose-response relationship for ultraviolet-light-induced mutations at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells. Somatic Cell Gen., 1: 383-389.

f. Rodent

(1) Specific Locus Assay in Mice

This test determines point mutations in mammals, a class of mutations of particular concern for human populations because the genetic damage may be initially invisible and only show serious consequences many generations later. Mouse coat color and morphologic markers representing seven specific loci are observed to estimate rates of forward mutation. After treatment wild-type males or females are bred with non-treated mates homozygous for the seven recessive genes; a viable mutation at any of the loci can be detected in the offspring allowing estimation of the rate of induction of recessive traits. This is a valuable test since it provides a direct measure of recessive mutations in mammals; it presently is the basis for most of the estimates of mutagenic risk in humans (Russell, 1951; Searle, 1975).

Advantages of this assay are: it is an intact *in vivo* system therefore different routes of exposure could be compared, and pharmacokinetic distribution mechanisms are operative; also most significantly, effects upon germinal tissue are being tested, and mutant animals can be visibly recognized at one week after birth. Disadvantages are that 20,000 to 30,000 offspring must be scored of each dose level of mutagen, making it a very expensive and time-consuming test; and that only a few loci are monitored and the nature of genetic alteration cannot be identified in a majority of cases. The test is considered marginally sensitive, and laboratories with the necessary mouse colony facilities and scientific expertise are limited.

(2) Mouse Isozyme Specific Locus Test

This is another murine specific locus assay involving mutations at characteristic isozyme loci - i.e., genes coding for variations in protein structure of different forms of the same enzyme. Specific enzymes from different tissues of offspring of treated male and female animals are compared for variation in electrophoretic mobility profiles (isoenzyme variance) as compared to the isozyme constitution of the same enzymes of the untreated parents. This analysis allows the detection of mutant phenotypes where the enzyme is not destroyed, but rather has had its charge altered due to changes in amino acid composition, or has undergone alteration in enzymatic activity. This assay requires selection of mouse strains with appropriate isoenzyme differences (e.g., C57BL/6 and DBA/2 mice) and is not yet as well developed as the preceding specific locus test (Valcovic and Malling, 1973).

V. B. 1. f. References for Gene Mutations - Rodent

(1) Specific Locus Assay/Mice

Russell, W. L. (1951). X-ray-induced mutations in mice. In: Genes and mutations, Cold Spring Harbor Symposia on Quantitative Biology, vol. XVI, pp 327-336. Cold Spring Harbor, L. I., New York. The Biological Laboratory.

Searle, A. G. (1975). The specific locus test in the mouse. Mutat. Res., 31: 277-290.

(2) Mouse Isozyme Specific Locus Assay

Valcovic, L. R. and Malling, H. V. (1973). An approach to measuring germinal mutations in the mouse. Environ. Health Perspect., 6: 201-205.

2. DNA Damage and Repair

These assays do not measure mutagenic activity as such, but indicate that a given chemical has entered the cell, and through some indeterminate mechanism caused damage to the DNA. One type of damage can be measured directly, i.e., by attempting to detect DNA single strand breaks. Other types of damage can be detected by testing for repair (by assay for nonreplicative synthesis) of that damage. Finally, biological systems which require repair of DNA for survival can be used to detect whether DNA damage has been caused by the treatment.

a. Repair-defective Microorganisms

(1) Recombinationless Mutants of Bacteria

Bacterial strains can be generated which have impaired mechanisms of DNA repair. The so-called "rec^- assay" involves screening chemical agents for relative toxicity against cells lacking this recombination capability (rec^-) and cells with this capability (rec^+). If the agent is more toxic against the former than the latter, DNA reactivity of the test chemical is indicated which is taken as indicative of a potential for mutagenicity of the test agent (Kada *et al.*, 1972; Nagao and Sugimura, 1972, Ichinotsubo *et al.*, 1977). These systems are currently being extensively validated in Japan.

(2) DNA Polymerase-deficient Mutants of Bacteria

Bacterial strains can be generated which are deficient in DNA polymerase (pol A^-), a DNA-synthesizing enzyme involved in repair processes. Bacterial cells exposed to agents that react with DNA can act to protect themselves by excising the altered DNA sequence and resynthesizing the correct sequence. Those bacterial strains which are polymerase deficient (pol A^-) are more sensitive to agents which modify DNA than those bacterial cells possessing the DNA polymerase (pol A^+). As in the case of the preceding assay, "rec" assay, this assay can be indicative of the potential for mutagenicity of a test agent, without employing a battery of tester strains or otherwise performing bacterial mutagenicity studies (Slater *et al*., 1971; Rosenkranz *et al*., 1976; Nagao and Sugimura, 1972).

(3) Normal versus Repair-deficient Yeast

Ultraviolet light-sensitive mutants of Saccharomyces cerevisiae can be generated and utilized as described above for sensitivity comparison with wild type cells as an indicator of the DNA reactivity of chemical agents (Nagao and Sugimura, 1972).

V. B. 2. a. References for Repair Defective Microorganisms

(1) Recombinationless Mutants/Bacteria

Kada, T., Tutikawa, K., and Sadaie, Y. (1972). *In vitro* and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutat. Res., 16: 165-174.

Nagao, M. and Sugimura, T. (1972). Sensitivity of repair-deficient mutants to 4-nitroquinoline 1-oxide, 4-nitropyridine 1-oxide, and their derivatives. Cancer Res., 32: 2369-2374.

Ichinotsubo, D., Mower, H. F., Setliff, J., and Mandel, M. (1977). The use of *rec*$^-$ bacteria for testing of carcinogenic substances. Mutat. Res., 46: 53-62.

Nishioka, H. (1975). Mutagenic activities of metal compounds in bacteria. Mutat. Res., 31: 185-189.

(2) DNA Polymerase-deficient Mutants/Bacteria

Rosenkranz, H. S., Gutter, B., and Speck, W. T. (1976). Mutagenicity and DNA-modifying activity: A comparison of two microbial assays. Mutat. Res., 41: 61-70.

Slater, E. S., Anderson, M. D., and Rosenkranz, H. S. (1971). Rapid detection of mutagens and carcinogens. Cancer Res., 31: 970-973.

Nagao, M. and Sugimura, T. (1972). Sensitivity of repair-deficient mutants and similar mutants to 4-nitroquinoline 1-oxide, 4-nitropyridine 1-oxide, and their derivatives. Cancer Res., 32: 2369-2374.

(3) Normal versus Repair-deficient Yeast

Nagao, M. and Sugimura, T. (1972). Sensitivity of repair-deficient mutants and similar mutants to 4-nitroquinoline 1-oxide, 4-nitropyridine 1-oxide, and their derivatives. Cancer Res., 32: 2369-2374.

b. Mammalian Cells

(1) DNA Single-strand Breaks

Several techniques are available for detecting single strand breaks in the DNA of treated cells; these include alkaline sucrose gradient centrifugation (Laishes and Stich, 1973; Stich *et al.*, 1975) and the alkaline elution technique (Fornace *et al.*, 1976; Kohn *et al.*, 1976; Swenberg *et al.*, 1976; Ewig and Kohn, 1977). The latter technique offers the additional advantage of detecting DNA-protein cross-links (Fornace and Kohn, 1976). It is important to determine the sensitivity of a given technique for the detection of single strand breaks. Attention should also be given, as for other mammalian cell assays, to the possible requirement for a metabolic activation system. Criteria for selection of a particular mammalian cell system for a given test material should be justified.

V. B. 2. b. References for DNA Damage/Repair - Mammalian Cells

(1) DNA Single Strand Breaks

Laishes, B. and Stich, H. (1973). Repair synthesis and sedimentation analysis of DNA of human cells exposed to dimethylnitrosamine and activated dimethylnitrosamine. Biochem. Biophys. Res. Commun., 52: 827-833.

Stich, H., Kieser, B., Laishes, R. and Warren, P. (1975). DNA repair of human cells as a relevant, rapid, and economic assay for environmental carcinogens. GANN Monogr. Cancer Res., 17: 3-15.

Fornace, A. J., Jr., and Kohn, K. W. (1976). DNA-protein cross-linking by ultraviolet radiation in normal human and xeroderma pigmentosum fibroblasts. Biochim, Biophys. Acta, 435: 95-103.

Fornace, A. J., Jr., Kohn, K. W., and Kann, H. E., Jr. (1976). DNA single-strand breaks during repair of UV damage in human fibroblasts and abnormalities of repair in xeroderma pigmentosum. Proc. Nat. Acad. Sci.,USA, 73: 39-43.

Kohn, K. W., Erickson, L. C., Ewig, R. A. G., and Friedman, C. A. (1976). Fractionation of DNA from mammalian cells by alkaline elution. Biochem. 15: 4629-4637.

Swenberg, J. A., Petzold, G. L., and Harbach, P. R. (1976). *In vitro* DNA damage/alkaline elution assay for predicting carcinogenic potential. Biochem. Biophys. Res. Commun., 72: 732-738.

Stich, H. F., Lam, P., Lo, L. W., Koropatnick, D. J. and San, R. H. C. (1975). The search for relevant short term bioassays for chemical carcinogens: The tribulation of a modern Sisyphus. Can. J. Genet. Cytol., 17: 471-492.

Ewig, R. A. G., and Kohn, K. W. (1977). DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. Cancer Res., 37: 2114-2122.

(2) Unscheduled (Nonreplicative) DNA Synthesis

Several techniques are available for detecting the occurrence of DNA repair, defined as unscheduled or nonreplicative DNA synthesis. These include: (a) incorporation of the DNA precursor ^{3}H-thymidine into the acid-precipitable DNA of the cells which are in G_0 stage (nonmitotic; nonproliferating, e.g., peripheral lymphocytes); (b) unscheduled DNA synthesis, wherein autoradiographic techniques are employed to detect cells which incorporate ^{3}H-thymidine into their DNA at a lower level during the labeling period than occurs in cells replicating their DNA. This technique detects repair synthesis in non-S phase cells; (c) repair replication; the technique involves alkaline density gradient certrifugation procedures to separate normal density repair-replicated DNA (labeled with ^{3}H-BrUdR or ^{3}H-thymidine plus nonradioactive BrUdR) from semi-conservatively synthesized BrUdR density-shifted DNA; and (d) photolysis of DNA incorporating 5-bromo-deoxyuridine into repair-replicated regions of the DNA.

The choice of cell system employed should be justified. The cell should either have its own metabolic capability for chemical activation, or one must be provided (e.g., normal human diploid cells with an *in vitro* metabolic activation system for unscheduled synthesis determination). At this time, the most extensive validation of any test exists for WI-38 system with metabolic activation (Simmon *et al*., 1977).

V. B. 2. b. References for DNA Damage and Repair - Mammalian Cells

(2) Unscheduled DNA Synthesis

San, R. H. C., and Stich, H. F. (1975). DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens. Int. J. Cancer, 16: 284-291.

Williams, G. M. (1977). Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell cultures. Cancer Res., 37: 1845-1851.

Simmon, V. F., Mitchell, A. D., and Jorgenson, T. A. (1977). Evaluation of selected pesticides as chemical mutagens: *In vitro* and *in vivo* studies. Annu. rep., Environ. Health Effects Series, EPA-600/1-77-028, May, 1977.

c. Rodent Germinal Cells (Mouse Spermatocytes)

Particularly relevant for test materials for which DNA repair is detected is the determination of whether or not the agent actually reaches germ cells and causes damage to the DNA of these cells. This can be accomplished by using nonreplicative DNA synthesis techniques, such as are described in the references given. Since one of the initial steps is the isolation and purification of sperm, a number of the above described DNA repair procedures might also be applicable. Whichever system is employed will require an appropriate data base, with adequate positive controls and all other in vivo criteria suitably specified (Sega et al., 1976).

V. B. 2. c. References for DNA Damage and Repair - Rodent Germinal Cells

Sega, G., Owens, J. and Cumming, R. (1976). Studies on DNA Repair in early spermatid stages of male mice after *in vivo* treatment with methyl-ethyl, propyl, and isopropyl methanesulfonate. Mutat. Res., 36: 193-212.

3. Chromosomal Effects

Chromosomal effects are defined as breaks within chromosomal structures (chromosomes or chromatids) with or without rejoining, or changes in chromosome number or any part of total morphology. It is important to differentiate between breaks and gaps, which may be unstable and heritable only to a slight degree; and chromosomal translocations and inversions (as well as other stable rearrangements), which are more likely to be heritable. Such induced chromosomal effects can be measured in a variety of test systems, ranging from microorganisms to the tissues of animals and man.

a. Yeast (Mitotic Recombination)

Mitotic recombination is defined as either reciprocal crossing-over or nonreciprocal gene conversion. The possible result of mitotic recombination is the generation of a new genotype by exchange of genetic material between homologous chromosomes followed by appropriate segregation of the products of mitosis. The result of such an event in a cell is the expression of a recessive phenotype in the homozygous condition which would otherwise go undetected in the heterozygous state (Brusick and Mayer, 1973).

Saccharomyces cerevisiae - Metabolic activation is required; test results may be dependent upon both time and pH.

b. Insect (Drosophila)

(1) Dominant Lethality

The measure of dominant lethal induction in Drosophila involves comparison of the percentage of eggs hatching in a control versus a treated series of pair matings. The failure of the zygote to develop beyond the embryonic stage is associated with the loss or gain of one of the major autosomes, or of large portions of such chromosomes or of the X chromosome. Since the presence of unhatched eggs can be due to factors other than chromosome breakage or chromosome loss (e.g., to direct effects on the gametes or to interference with the mating performance of the flies), the occurrence of a positive test result in the very simple Drosophila dominant lethal test should be followed by other available genetic tests in Drosophila.

(2) Chromosomal Rearrangements

Tests for chromosomal rearrangement would not necessarily be performed as an initial test, but rather as a confirmatory test for dominant or recessive lethal positives in Drosophila. This would allow a determination as to whether the mutagen is acting by point mutations or by breaking chromosomes as well.

Available tests include genetic tests for reciprocal translocations, position-effect tests for chromosomal rearrangement, and cytological tests for chromosomal rearrangement.

V. B. 3. References for Chromosomal Effects

a. Yeast

Brusick, D. and Mayer, V. (1973). New developments in mutagenicity screening techniques with yeast. Environ. Health Perspect., 6: 83-96.

Zimmerman, F. (1975). Procedures used in the induction of mitotic recombination and mutation in the yeast *Saccharomyces cerevisiae*. Mutat. Res., 31: 71-86.

Zimmerman, F. (1973). Detection of genetically active chemicals using various yeast systems. In: Chemical mutagens, Principles and methods for their detection, vol. 3, A. Hollaender, ed., pp 209-239. New York. Plenum.

Mortimer, R. and Manney, T. (1971). Mutation induction in yeast. In: Chemical mutagens, Principles and methods for their detection, vol. 1, A. Hollaender, ed, pp 289-310. New York. Plenum.

b. Insect

(1) Dominant Lethality

Abrahamson, J. and Lewis, E. (1971). The detection of mutations in *Drosophila melanogaster*. In: Chemical mutagens, Principles and methods for their detection, vol. 2, A. Hollaender, ed., pp 461-487. New York. Plenum.

Vogel, E. and Leigh, B. (1975). Concentration-effect studies with MMS, TEB, 2,4,6-triCl-PDMT, and DEN on the induction of dominant and recessive lethals, chromosome loss and translocation in *Drosophila* sperm. Mutat. Res., 29: 383-396.

(2) Chromosomal Rearrangements

See references for (1) Dominant Lethality above.

c. Mammalian Cells

(1) Chromosomal Aberrations

Mammalian cells in culture provide an in vitro model system for assessing chromosomal damage, which may also be indicative of the potential of the agent for inducing gene mutations. The lesion can be classified as a chromatid lesion involving only one of the two chromatids of the chromosome or a chromosomal lesion in which both chromatids are involved. A chromatid lesion is likely to occur if damage occurs after the DNA of that chromosome has been replicated, while a chromosomal lesion results when damage occurs before the chromosome had replicated its DNA. Abnormalities seen include breaks, gaps, and rearrangements, with the latter requiring continual cellular metabolic function to occur. Stable effects such as deletions and translocations are considered most significant.

In vitro cytogenetic studies of mammalian cells can employ either continuously proliferating cell lines or freshly isolated peripheral leukoyctes from a variety of species. If continuous cell lines are employed, the cytogenetic character of the cell line should be well documented, and previous experience of effects of other agents, including x-irradiation, should be available. The ploidy must be constant.

These cell lines can be exposed either as an asynchronous, rapidly proliferating population, where collection and cytogenetic analysis of the cells at the first mitosis after treatment results in indication of chromosome damage induced at all stages of the cell cycle; or they can be exposed as a synchronized population of cells, where the tester must indicate why he has so chosen specific stages (e.g., increased sensitivity) for testing.

When leukocytes are treated prior to mitogen stimulation, the cytogenetic analysis of the cells, if performed at the first mitosis after treatment, will be indicative of chromosome damage induced pre-DNA synthetic (S) period. The tester can alternatively choose to treat with test agent during the period of mitogen stimulation.

Criteria for selection of cell line (and species), protocol for treatment, protocol for collection of mitotic figures, and protocol for analysis and scoring and for statistical evaluation should be specified. He should state how his controls were handled, and how the experiments were repeated. It is also important to employ "double-blind" studies to avoid observer bias, and to describe test controls and replications.

V. B. 3. References for Chromosomal Effects (continued)

c. Mammalian Cells

(1) Chromosomal Abberations

Cohen, M. and Hirschhorn, K. (1971). Cytogenetic studies in animals. In: Chemical mutagens, Principles and methods for their detection, vol. 2., A. Hollaender, ed. New York. Plenum.

Brewer, J. and Preston, R. (1974). Cytogenetic effects of environmental mutagens in mammalian cells and the extrapolation to man. Mutat. Res., 26: 297-305.

Nichols, W. (1973). Cytogenetic techniques in mutagenicity testing. Agents Action, 3: 86-92.

Ishidate, M., Jr. and Odashima, S. (1977). Chromosome tests with 134 compounds on Chinese hamster cells in vitro - a screening for chemical carcinogens. Mutat. Res., 48: 337-354.

(2) Sister Chromatid Exchange

Sister chromatid exchange (SCE) involves a symmetrical exchange at one locus between sister chromatids, which does not result in an alteration of overall chromosome morphology.

The measurement of sister chromatid exchanges can be performed with proliferating mammalian cells in culture; the availability of fluorescent techniques eliminates the need for radioactivity. The procedure requires less time and skill than analysis and scoring of chromosome aberrations, but it is important to remember that this test involves cells in the mitosis of the second cell cycle after initiation of treatment and therefore selects for only those treated cells which can pass through the first mitosis and all of the second cell cycle. Chromosome alterations, in contrast, can be investigated at the first and/or second mitosis. The occurrence of SCE seems to be related more closely, when different chemical mutagen effects are compared, to chromosomal abnormalities in eukaryotes than to induction of point mutations in bacteria. The sensitivity of SCE appears to be greater for some chemicals than morphological chromosome damage detection, but SCE may not be as responsive as the latter to other chemicals.

SCE can be detected using either continuous cell lines or freshly-isolated peripheral lymphocytes, which are cultured _in vitro_ and mitogen stimulated. It is important to specify: criteria for selection of cell system (and/or species); protocol for treatment; and protocol for analysis and scoring. Analysis should be performed using "double-blind" procedures.

V. B. 3. References for Chromosomal Effects (continued)

c. Mammalian Cells

(2) Sister Chromatid Exchange

Perry, P. and Evans, H. J. (1975). Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature, 258: 121-125.

Stetka, D. G. and Wolff, S. (1976). Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. II. *In vitro* test for compounds requiring metabolic activation. Mutat. Res., 41: 343-350.

Stetka, D. G. and Wolff, S. (1976). Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. I. *In vivo* test for compounds requiring metabolic activation. Mutat. Res., 41: 333-342.

Taylor, J. (1958). Sister chromatid exchanges in tritium-labeled chromosomes. Genet., 43: 515-529.

Perry, P. and Wolff, J. (1974). New giemsa method for the differential staining of sister chromatids. Nature, 251: 156-158.

Latt, M. (1976). Analysis of human chromosome structure, replication and repair using Brd V-33258 Hoechst techniques. J. Reprod. Med., 17: 41-52.

Solomon, E. and Borrow, M. (1975). Sister chromatic exchanges - a sensitive assay of agents damaging human chromosomes. Mutat. Res., 30: 273-278.

d. Rodents

(1) Cytogenetic Analysis

In performing cytogenetic analysis upon *in vivo* exposure, three systems are of primary interest. These are the cells of the bone marrow, circulating peripheral lymphocytes, and spermatocytes (after exposure as spermatogonia). In performing *in vivo* cytogenetic tests, attention should be given to selection of species (sensitivity, number and morphology of chromosomes, pharmacology), spontaneous incidence of chromosome aberrations, effects of age, effects of other environmental factors, optimum time of examinations, and duration of treatment. In performing these tests consideration should be given to application of SCE studies and the micronucleus test.

Bone Marrow

While proposed herein for studies of induction of chromosome and chromatid aberrations, the cytogenetic analysis of bone marrow is the best source of information dealing with direct effects on mitosis (Mitotic index); no *in vitro* culture is involved. Such studies can be performed after gaseous exposures.

Circulating Peripheral Lymphocytes

Circulating peripheral lymphocytes provide the best system for showing effects of *in vivo* exposure on cells in G_1, since most of these cells are in the pre-DNA synthetic phase while in circulation. The study of aberration induction in these cells offers great potential for use during chronic exposure since the exposed animals need not necessarily be terminated to perform this analysis. Tests can also be performed after (or during) gaseous exposure.

Special attention should be given to the time of analysis after mitogen stimulation, i.e. 48 vs. 72 hours, so as to distinguish cells in first mitosis from those in second.

Spermatocytes

The study of gametocytes can be of considerable importance, since it is the only means to detect potential genetic damage without actually studying the offspring.

In performing these tests, it may be necessary to investigate a complete cycle of spermatogenesis so as to include effect differences on specific stages.

(2) Heritable Chromosomal Damage

Sex Chromosome Loss

This test can be performed in Drosophila, with treatment of the male parent being performed. The test available should be performed with caution, however, since the spontaneous frequency of loss of a given chromosome is much more subject to external modification than is the spontaneous frequency of mutation of a given gene. In performing this study, F_1 progeny of treated males are scored for entire sex-chromosome loss and for partial loss of the Y chromosome.

Dominant Lethal Effects

Dominant lethal mutations can be used as indicators of major genetic damage in mammals. The genetic basis for dominant lethality is mainly the induction of structural and numerical chromosomal anomalies, such as translocation and aneuploidies. These in turn may induce a) preimplantation losses of nonviable zygotes; b) early fetal deaths; and c) sterility in F_1 progeny.

The advantages of this procedure are that a) the test organism is a mammal and therefore has some biochemical and metabolic resemblances to man; b) the test cell is a germ cell; and c) the results are obtained before the first generation is born. Disadvantages are: a) there is no definitive proof that a given lethal embryo is killed by genetic damage; b) the type of damage is chromosomal, which may not be related to gene mutation; c) the damage in evidence will most likely only be related to post-meiotic stages of spermatogenesis, even if earlier stages are damaged; and d) the limitations of the biochemical and pharmacological relationship of the mouse to man.

It is important to specify exposure protocol and state criteria for the selection of mode of treatment. The schedule of matings will have to be specified, as will the criteria for choosing a given mating schedule. The data recorded can include a) the number of pregnant females per number of mated males; b) the total number of implantations; c) the total number of corpora letea (preimplantation losses); d) dead implantations; e) proportion of females with either one or more, or two or more, dead implantations; and f) dead implantations per total implantations.

Criteria for the data recorded and presented should be indicated.

Heritable Translocation Test

The heritable translocation test can be differentiated from the spermatocyte test on treated males in that the assay is performed by testing for sterility and semi-sterility in F_1 male progeny of the treated male mice. After confirmation by a second mating of the semi-sterility or sterility of the F_1 male progeny, the primary spermatocytes of the latter are analyzed cytogenetically to confirm the presence of a heritable chromosome translocation.

The evidence currently available indicates that many translocations occurring prior to meiosis may be eliminated by this process, which makes the F_1 translocation assay valuable primarily for measuring effects induced in postmeiotic stages of spermatogenesis.

The major advantage of the heritable translocation assay is that it measures an explicit genetic event, with possible limitation to assessment of postmeiotic chromosomal damage. Live progeny are observed, another advantage, but a large number of animals are required and a six-month period is necessary to complete the assay. The cost per assay is also very high, while response to known mutagens may be low.

The mouse appears to be the species of choice. In general, translocation heterozygotes are detected by testing for sterility and semi-sterility in F_1 male progeny of treated male mice. Generally, two or three females are mated with each F_1 male and necropsied at mid-term to determine the number of viable embryos. Those males that produce a significant reduction in the number of living embryos are then remated to another series of females for additional evidence of semi-sterility. Finally, semi-sterile and sterile males are examined cytogenetically by analyzing primary spermatocytes to confirm the presence of a heritable chromosome translocation.

V. B. 3. References for Chromosomal Effects (continued)

d. Rodents

(1) Cytogenetic Analysis

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V. B. 3. d. (continued)

(1) Cytogenetic Analysis (continued)

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(2) Heritable Chromosomal Damage

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V. B. 3. d. (continued)

(2) Heritable chromosomal damage (continued)

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4. Methodological Considerations

a. Metabolic Activation

The need for metabolic activation of pre-carcinogens to an ultimate carcinogenic form is well documented. In attempting to perform any type of bioassay, one must first determine if the system being employed as the indicator has its own inherent capability to perform this metabolic activation, and secondly, if it can achieve a sufficient amount of activation during the period of the treatment with the test agent. Related to the latter is the question of whether or not the necessary activation system is pre-inducible in the test system to obtain higher levels of activity.

If the system does not possess its own metabolic activation system, then an appropriate mechanism to allow for the necessary activation must be included in the assay. Metabolic activation for *in vitro* assay systems will generally require the use of human or animal tissue. Exceptions may be *Drosophila* and plant (*Tradescantia*) systems. Attention should also be given to the factors of species, strains, age, sex, and tissue of origin of metabolically capable preparations.

(1) *In vivo*

Considering the necessity for metabolic activation/ deactivation of test materials, one approach is to employ the whole animal as the metabolic factory. The route of administration can be varied, and the chemicals will be metabolically activated (or detoxified) in those tissues in which they localize and which have the enzymatic capability.

Two avenues for testing are then available. The first is to collect urine from treated animals, and after appropriate treatment of the urine to deconjugate potentially active metabolites, to then treat the test organisms with the urine. The second approach, known as "host-mediated assay", involves locating the test organism or cell in particular tissues or organs of an animal, treating the whole animal with the test agent (using various routes of administration), and recovering and subsequently assaying *in vitro* for a defined endpoint effect. This procedure has been employed with bacterial, yeast, and mammalian cell tests.

It will be necessary to consider the best currently available information, and to specify criteria for selection of species, strain(s), age(s), sex(es), and tissue of the animal employed. Attention must be given to the availability of a data base for the selected system, and to insure that the activation system is pre-induced to a high metabolic capability.

(2) *In vitro*

In addition to all of the considerations described above, the tester can select from a variety of *in vitro* metabolic activation systems that are potentially useful, including: 1) the S-9 fraction; 2) isolated microsomes; 3) postmitochondrial supernatant; 4) tissue homogenate; or 5) a mammalian cell feeder layer. Each of these systems has advantages and disadvantages; which should be considered in relation to the endpoint indicator organism being employed. It is important to include positive known controls in all tests in order to ensure that the test material has not inactivated the metabolic activation system.

b. Treatment Conditions

It is realized that the test materials pertinent to fuels and fuel additives will generally not be soluble in aqueous solutions and may be complex mixtures of variable and indeterminant chemical composition. It will therefore be essential that every effort is made to ensure that all components of a given test material actually do contact the cells of the test organism and are taken up by the cells.

These considerations are particularly important for *in vitro* tests with unicellular organisms. For bacterial systems, spot tests or plate tests can be performed, but neither will be satisfactory if the chemical is insoluble in aqueous medium, and cannot diffuse through semisolid agar during the continuous exposure period. Spot tests for mutagenicity are simplest, but would only be recommended for strong or volatile mutagens, since only a minority of cells on the plate would be exposed to an effective level of mutagen. Suspension treatmment of bacteria otherwise appear to be required (with and without metabolic activation), but consideration should be given to the relative shortness of exposure time in suspension treatments as compared to the plate methods; i.e., can enough chemical be metabolized to an active form during a short exposure period to provide a high enough concentration of activated ingredient to produce a significant effect. Volatile components or gaseous exposures require additional considerations, and methodological variations of the best available design should be employed.

In general, for any procedure, a major consideration is the effective level of the mutagen to be employed. If the concentration of a mutagen is too low, too few mutants will be induced to be detected. If the concentration is too high, too many mutants will be killed for any to be detected. The test concentration should not exceed 90% killing, and should in general range from 0-90%. Further discussion of the various techniques for bacteria can be found in the references listed.

The use of spot test procedures or treatment in suspension are also employed for studies with *Saccharomyces cerevisiae*, with the above considerations being relevant; forced heterokaryons of *Neurospora crassa* are usually treated in suspension. For mammalian cells, the treatment protocol employed may have to be varied from that recommended in the references provided in the appendix; for example, chemical transformation experiments generally allow for a continuous 72 hours of treatment; for mixtures, this might have to be prolonged. The standard protocol for at least one mammalian cell mutagenesis assay is defined as two hours; this also may have to be prolonged. Appropriate judgments will have to be made as to length of exposure versus linearity of microsomal activation *in vitro* when the latter is required in conjunction with mammalian cell assay system. Modifications for gaseous exposure will have to be developed.

Requirements for utilization of specific bacterial test systems for detection of mutagenic activity are not detailed in these draft protocols; and indeed, the variety and usefulness of these systems is continually expanding, so that specific selection guidelines will require informed judgmental decisions. However, attention is directed to one bacterial system which might have unique application for fuels and fuel additives. This is the *Escherichia coli* K12 system developed by Mohn *et al*. (1974, 1975), which is unique in that it manifests greater sensitivity towards certain carcinogens, such as nitroheterocyclic compounds and dialkylnitrosamines than do the *Salmonella* strains. In addition, this *E. coli* strain allows for the forward detection of several genes (whereas *Salmonella* systems detect only reverse mutations) and is more suitable for the intra-sanguineous host-mediated assay (Sobels, 1977).

For *Drosophila*, which is an *in vivo* system, considerations must be given to the possibility that the flies can voluntarily close their spiracles, and therefore prevent entry of a chemical in aerosol form.

V. B. 4. References for Methodological Considerations

(a) Metabolic Activation

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(b) Treatment Conditions

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V. B. 4. References for Methodological Considerations

(b) Treatment Conditions (continued)

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V. C. Carcinogenesis

1. Selected Mutagenesis Tests
2. Mammalian Cell Neoplastic (Oncogenic) Transformation
    a. Primary Cells
    b. Continuous Lines
3. Rodent Tests
    a. Pulmonary Tissue Carcinogenesis
        Aerosol inhalation
        Vapor inhalation
        Intratracheal instillation
    b. Non-pulmonary Target Tissue Carcinogenesis
        Pulmonary exposure
        Skin painting
        Carcinogen bioassay - oral administration
4. Cocarcinogenesis and Tumor Promotion
    a. Mouse Skin Papilloma
    b. C3H/10T½ Mammalian Cells in Promoter Mode

V. C. Carcinogenesis

1. Selected Mutagenesis Tests

Chemicals which are either carcinogenic or mutagenic are capable of entering cells and reacting or interacting with DNA. If the cell within which the chemical "hits" the DNA is a germ cell, mutagenesis may be induced, while if the involved cell is a somatic cell, carcinogenesis may result. The potential for damage to DNA which characterizes both mutagens and carcinogens can be employed to advantage for rapid and inexpensive *in vitro* screening of environmental chemicals. The quickest and least expensive of these *in vitro* screening tools are the bacterial mutation detection assays, which have an impressive record for predicting carcinogenic potential when coupled with mammalian metabolic activation systems. For evaluation of fuel and fuel additive test materials for which the primary route of exposure is inhalation and pulmonary carcinogenesis is a priority concern, it may be advisable to consider utilization of both mammalian lung and liver activation systems in order to account for any significant differences in metabolic toxification or detoxification capacity between the two organs.

The current status of the utilization of bacterial mutagenesis assays for detection of carcinogenic potential has recently been reviewed (Bridges, 1976).

2. Mammalian Cell Neoplastic (Oncogenic) Transformation

For purposes of short-term, *in vitro* predictive screening for carcinogenic potential in a mammalian system, mammalian cells in culture have been used. The endpoint sought is transformation to a malignant state by treatment with a test material. The only absolute indicator for this endpoint is the ability of cells to produce a tumor when inoculated into an appropriate host animal; however, there are various secondary growth characteristics which correlate with malignancy, and can be visually monitored in culture. Transformed cells may have a number of distinguishing characteristics, including: 1) morphological alteration; 2) ability to form colonies in a semi-solid medium without anchorage; 3) ability to form multilayered clones under conditions where normal cells remain as a monolayer; 4) ability to form clones on a monolayer of normal cells; 5) ability to grow in low concentrations of serum; 6) ability to grow more rapidly than non-transformed cells, particularly in low concentrations of serum; 7) ability to survive under conditions where normal cells tend to senesce and die ("immortalization"); 8) presence of altered membrane properties, including lectin binding and lectin-induced agglutination, etc. (Martin and Anderson, 1976; Steuer *et al*., 1977). This list is not inclusive; biochemical indicators such as the ability to secrete a proteinase involved in fibrinolysis and the release of plasminogen activators can also be distinguishing markers, and other markers are continuously being developed. Most important, however, is the demonstration and documentation, for whatever endpoint of transformation is utilized, that the selected endpoint correlates with the generation of tumors in animal hosts upon injection of transformed cells.

V. C. 2. a. Primary Cells

Primary or secondary cultures of cells from Syrian hamster embryos can be transformed quantitatively by exposure to carcinogens in culture. Transformation in culture as judged by morphological parameters correlates with the production of sarcomas when the cells are inoculated into hamsters (DiPaolo *et al*., 1971, 1972).

Good correlations have been obtained between carcinogenic activities of various compounds and their ability to transform these cells in culture (Heidelberger, 1975; and references therein). A recently published study describes a 90.8% positive correlation for 82 chemicals known to be either carcinogens or non-carcinogens (Pienta *et al*., 1977). These cells have the capacity to metabolically activate some classes of carcinogens such as polycylic hydrocarbons and alkylating agents, although other carcinogen classes are not activated by early passage cultures - i.e., primary or secondary cultures (Pienta *et al*, 1977; DiPaolo *et al*., 1972). For screening of fuel and fuel additive test materials, it would therefore be advisable to screen test materials both with and without mammalian metabolic activating systems. This assay discriminates between structurally related carcinogens and noncarcinogens; no false positives were obtained when noncarcinogens were assayed; and the system is sensitive to metal carcinogens (Pienta *et al*., 1977). Methods for optimizing and standardizing this assay for *in vitro* carcinogenesis screening are outlined by Pienta *et al*. (1977) and Casto *et al*. (1977).

V. C. 2. a. References for Primary Cells

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V. C. 2. b. Continuous Lines

Continuous cell lines have been derived from rodent embryo cells, offering the advantage of working with a homogeneous cloned cell population rather than the heterogenous population of primary cell cultures. Again, good correlations have been observed between carcinogenic activity and the extent of transformation produced in cultured lines. The lines specified here have been shown to exhibit good stability in culture and a high degree of sensitivity to post-confluence inhibition of division (Reznikoff *et al*., 1973a, 1973b). The capacity for metabolic activation of carcinogens of various chemical classes varies from one clone to another for a given cell line and probably as a function of length of passage in culture also. It would therefore be wise to screen fuel and fuel additive test materials both in the presence and the absence of supplementary mammalian metabolic activating systems.

Other rodent cell lines have been employed for detection of oncogenicity in culture, specifically rat embryo cell lines (Sekely *et al*., 1973; Rhim and Huebner, 1973) and a baby hamster kidney line, BHK_{21} (Di Mayorca *et al*., 1973).

Mouse embryo fibroblast line, C3H/10T½/CL8
This system has been characterized (Reznikoff *et al*., 1973 a, 1973b) and modified for two stage oncogenesis (Mondal *et al*., 1976).

Mouse embryo fibroblast line, BALB/3T3, clone A31-1
Application of this system to the assay of carcinogenic activity has been described by Kakunaga (1973, 1974) and DiPaolo *et al*. (1972).

V. C. 2. b. References for Continuous Lines

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V. C. 3. Rodent

a. Pulmonary Tissue Carcinogenesis

Motor vehicle emissions are known to contain a number of compounds which act as chemical carcinogens when tested in animals. These emission components include all three types of chemical carcinogens:

(1) direct-acting or primary carcinogens, such as aryl epoxides, alkylene epoxides and lactones and sulfate esters;
(2) secondary or procarcinogens, such as benz(a) anthracene and benz(a)pyrene; and
(3) co-carcinogens or promoters, such as phenols and glycols.

Primary carcinogens frequently act to produce tumors at the points of application since they do not require metabolic activation, although they are subject to detoxification and excretion; however, they may also act in remote tissues. Secondary carcinogens usually do not act at the point of application, but often involve specific target tissues; they *require* metabolic activation, but also are detoxified. Cocarcinogens are not carcinogenic alone, but can markedly potentiate carcinogen action. They do not necessarily have to be administered simultaneously with, or even by the same route as the carcinogen. A comparatively larger amount of cocarcinogen can promote tumors from an extremely small amount of carcinogen. This promoter effect may be very important for human exposure to motor vehicle emissions as the carcinogenicity of exhaust condensate has been found to be greater than can be accounted for on the basis of known carcinogen concentration (Van Duuren, 1976; Hoffman and Wynder, 1976).

Inhalation exposure is characterized by: (1) large intake volumes; (2) repetitive intake; (3) large tissue surface area in rapid equilibrium with the entire blood circulation; (4) ability of respiratory tract tissues to metabolically activate secondary carcinogens; and (5) specific binding and retention by lung tissue of certain chemical classes of potential carcinogens such as hydrophobic amines (Philpot *et al.*, 1977). Not only are hydrophobic amines concentrated in lung, regardless of the route of administration, but lung tissue has a capacity for N-oxidation of such amines that is equal to that of liver, in spite of a lower concentration of P-450 than the liver (Brown, 1974). However, N-hydroxylation is thought to be the major pathway by which aromatic amines and their nitro derivatives are converted to the ultimate carcinogen form and the occurrence or concentration of this enzyme activity in various organs may explain why aromatic amines are predominantly bladder carcinogens. The metabolism of aromatic amines, and their subsequent carcinogenicity are affected by hormonal changes also (Clayson and Garner, 1976).

Specific lung carcinogens include bis (chloromethyl) ether (BCME). BCME, a vapor thought to be formed from the reaction of formaldehyde and hydrochloric acid, produced a high incidence of lung cancer in rats exposed one time to 0.1 ppm BCME. The powerful lung-specific carcinogenicity for humans of BCME has been confirmed by epidemiological evidence from chemical industry workers exposed to BCME and commercial chloromethyl ether which contains 1-7% BCME (Parkes, 1976).

In considering the capacity of test emissions for contributing to the incidence of cancers of the respiratory tract in human populations, it is important to evaluate:

primary carcinogenicity;
secondary carcinogenicity;
(after metabolism)
and cocarcinogenicity;
(direct and two-stage).

The effects of sex, hormonal status, diet, age, and organ-specific metabolic activation/deactivation should also be considered.

Aerosol inhalation

This is the preferred method of exposure because it parallels the human exposure situation, and is therefore essentially free of experimentally-introduced artefacts when whole exhaust is the test material. Solid fractions of exhaust may also be aerosolized with due experimental caution. Complete pathological study of the entire respiratory tract should be performed.

Vapor inhalation

If whole exhaust is not employed as the material for aerosol inhalation, then the carcinogenicity and cocarcinogenicity of the vapor phase should be evaluated.

Intratracheal instillation

Intratracheal instillation is a useful method of exposure for those cases where: the amount of test material (liquid or solid) is limited; or the test material is known or suspected to be toxic, flammable, explosive, or otherwise hazardous.

V. C. 3. b. Non-pulmonary Target Tissue Carcinogenesis

Pulmonary exposure
The incidence of tumors at sites other than in the respiratory tract as a result of pulmonary exposure should be observed. This would logically be combined with the evaluation of respiratory tract carcinogenesis.

Skin painting
The potential of a given test material for carcinogenicity may be evaluated by the application of solutions of the test material in an appropriate nontoxic solvent to mouse skin. This assay should be coupled with one which screens the carcinogenic potential of the vapor-phase of emissions.

Carcinogen bioassay - oral administration
The test material may be administered by gavage (stomach tube), preferably; or mixed into food and the amount consumed monitored.

All significant tissues, including those of the respiratory tract, should be examined pathologically as detailed in the NCI Guidelines for Carcinogen Bioassay in Small Rodents (Sontag, Page, and Saffiotti, 1976), along with other parameters of chronic toxicity. If any fuel or fuel additive test materials are evaluated for chronic toxicity by any route of administration, this carcinogenesis bioassay should also be included. It would also be advisable to consider observation of all animal test groups for purposes of determining differences in time-to-tumor development as well as differences in tumor incidence (Hoel *et al*., 1975).

V. C. 3. References for Carcinogenesis - Rodent

a. Pulmonary Tissue Carcinogenesis

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V. C. 4. Cocarcinogenesis and Tumor Promotion

Cocarcinogenesis is the augmentation of tumor production over that produced by the carcinogen alone. If the test substance is applied at the same time as the carcinogen and enhancement of tumor production is observed, the substance is considered a cocarcinogen. If the test substance produces an increase in tumorigenesis when administered subsequently to the carcinogen, this is termed promotion, or two-stage carcinogenesis. Promotion may occur if the promoter is administered through a different route than the carcinogen. For screening of fuels and fuel additives health effects, cocarcinogenesis occurring in the respiratory tract is of greatest interest, and some studies have dealt with this (Mohr *et al*., 1976); however, the most widely employed and best characterized system for studying cocarcinogenesis is that employing mouse skin (Berenblum and Shubik, 1949; Van Duuren, 1967). An *in vitro* assay for promoter activity has recently been described (Mondal *et al*., 1976).

Appropriate forms of test material should be screened for cocarcinogenic and tumor promotion activities with one or more of the polyaromatic hydrocarbon carcinogens known to occur in motor vehicle emissions. If only one carcinogen is employed, it should be that occurring in highest concentration in emissions.

a. Mouse Skin Papilloma (Two-stage Oncogenesis)

An assay for tumor promotion activity, which is defined as enhancement of tumor formation by low doses of a carcinogen due to subsequent action of the promoter, has been described by Berenblum and Shubik (1949).

In addition to tumor promoters, a number of chemicals such as various aliphatic hydrocarbons have been shown to be cofactors for carcinogenesis, markedly enhancing carcinogenicity when applied to mouse skin simultaneously with a carcinogen (Van Duuren, 1976; Weinstein and Troll, 1977).

Fuel and fuel additive test materials should be evaluated as both promoters and cofactors for carcinogenesis.

b. C3H/10T½ Mammalian Cells in Promoter Mode

It has recently been demonstrated that substances which act as tumor promoters *in vivo* also display promoter activity *in vitro* by enhancing transformation of C3H/10T½, a mouse embryo cell line, when administered 4 days after a non-transforming dose of carcinogen (Mondal *et al*., 1976).

V. C. 4. References for Cocarcinogenesis and Tumor Promotion

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V. D. Teratogenesis and Reproductive Performance

1. Reproductive Performance

2. Teratogenesis

V. D. Teratogenesis and Reproductive Performance

1. Reproductive Performance

Reproductive performance is concerned with any action of an environmental agent at any time relative to the conception process which can affect the ability of parents to successfully produce offspring, and would include effects occurring during the reproductive cycle(s).

In performing studies to evaluate the effects of long-term, continuous, low-dosage exposures on overall reproductive integrity, the so-called "three generation test" recommended by the Food and Drug Administration appears appropriate. These tests provide an opportunity for accumulation of substances or insults to the point where deleterious effects may be observed. As described by the Food and Drug Administration Advisory Committee on Protocols for Safety Evaluations (1970), the study can give information on fertility and pregnancy in the parent F(0) generation. "Observations of the F(1) generation would determine the effects of a substance upon the uterine environment and upon lactation as well as on postweaning growth and development. The F(1) animals which have been exposed continuously to this substance from the time of conception would reveal changes in reproduction characteristics acquired during the periods of embryogenesis, infancy, puberty, and reproductive maturity. The accumulation of a potentially toxic substance would be determined by the reproductive performance of the F(1) generation and observation of the growth and development of the F(2) generation." Apparently, some question exists as to the information to be gained on studying an additional F(3) generation.

Descriptions of current methodology and critiques of these procedures can be found in the references. Attention should be given to criteria for selection of species, number of species, strain, exposure, and especially to selection of endpoints for evaluation of effect and statistical analysis of the effect. The numbers of animals must be selected with attention to the latter.

2. Teratogenesis

Teratogenesis is concerned with the occurrence of developmental errors, both structural and functional, which are initiated at any time between fertilization and postnatal maturation. Because teratogenic effects can be masked in a variety of ways during a continuous exposure (e.g., by chemical alteration in the parent of metabolism of the treatment chemical), establishment of teratogenic risk should be investigated by short-term exposure during a period of high susceptibility. The embryonic period when early organ formation is in progress appears to be the time when the developing organism is most sensitive to adverse influences; it is recommended, however, that attention also be given to exposure during late pregnancy.

Descriptions of current methodology and critiques of these procedures can be found in the references listed. Attention should especially be given to specification of criteria for the selection of species and number of species, as well as exposure levels, route(s) of exposure, times and intervals of exposure, and for endpoints for evaluation of effect. The numbers of animals studied and procedure for statistical analysis should be justified.

V. D. References for Teratogenesis and Reproductive Performance

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2. Teratogenesis

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VI. POSSIBLE TESTING APPROACHES

A. Matrix Approach

B. Heirarchical Approach

VI. POSSIBLE TESTING APPROACHES

With the increasing realization that human exposure to chemicals may contribute to a significant health risk, federal agencies and other groups have formulated recommendations and guidelines regarding evaluation of these chemicals and the health risk they may present. Guidelines have been published for the testing of pesticides, food additives, and household substances. Recent concern regarding the increased incidence of cancer and potential genetic hazard to future generations due to mutagenic and carcinogenic chemicals in the environment has led to recommendations for mutagenicity-carcinogenicity screening of chemicals. These guidelines and recommendations for health effects testing of chemicals can be classified as involving either a matrix (battery) approach or a hierarchical (tier) approach. Either of these approaches could be implemented with a fixed set of tests or could employ a flexible set of tests which would be selected based on the chemical structure of the test material, pre-existing data base and other weighting factors discussed in Chapter III.

Examples of these two approaches are shown in Tables 1 and 2. The particular tests listed in each table are shown only as examples. The final determination of which specific tests and approach to testing will be implemented would take into account many of the following factors:

1. The purpose of such tests (e.g., to detect potential biological activity versus making regulatory decisions).

2. The weighting factors discussed in Chapter III.

3. Consultation by scientific experts as to which tests are most appropriate.

4. Desirability for uniform health effects testing requirements within EPA and across several regulatory agencies.

5. The number of fuels and fuel additives or test materials derived from them that would require testing.

6. The cost/benefit considerations for including specific tests.

A. Matrix Approach

The matrix approach to health effects testing which, for example, is utilized in the registration of pesticides, involves the employment of a prescribed battery of tests which may be sufficient for human risk assessment. Under these guidelines all tests are completed prior to decision making. Generally acute tests are utilized in this approach to determine exposure conditions for the chronic studies. The matrix of tests employed can be flexible as shown in the example (Figure 1, mutagenicity column) where 8 tests must be completed from three different areas, but there is some choice in the specific test employed. Alternatively, the entire matrix could be fixed such that a specified number of tests would be required for registration of the substance.

The matrix approach to health effects testing has been widely used and accepted as an approach which permits safety evaluation. This approach is most applicable to the evaluation of relatively small numbers of chemicals which are expected to present a significant human exposure, as in the case of drugs, food additives and pesticides. This approach has not been used with combustion products nor have the matrices previously employed emphasized inhalation exposures which would be most useful in a health risk assessment of fuels and fuel additives. The matrix approach would be the most definitive as well as the most expensive approach to testing fuels and fuel additives.

B. Heirarchical Approach

The heirarchical approach to health effects testing has been recommended as an approach to testing large numbers of compounds. A tiered battery of tests is employed in a step-wise fashion with results from each of the first tier of screening tests determining whether further tests should be used. In this approach, after prioritizing chemicals based on chemical structure (Tier 0), a large number of chemicals would be screened in rapid, sensitive *in vitro* or acute *in vivo* tests. The first tier or level of tests detects biological activity in the areas of toxicity, mutagenicity, carcinogenicity, and teratogenicity. The second tier of testing employs confirmatory or subchronic tests while the third tier of tests involve chronic whole animal exposures which should allow risk assessment.

It is important that the tests in the first tier detect as few false negatives as possible since confirmation (Tier 2) tests will generally be employed only on those test materials detected as potentially hazardous. This approach therefore tests larger numbers of compounds in the earliest tiers with less expensive and less time consuming tests. Only a small percentage of the compounds in Tier 1 would be expected to show sufficient biological activity to warrant Tier 2 or 3 testing. The later (higher) levels of tests are more time consuming and expensive. The entire tier would need to be completed prior to a human risk assessment.

VI. References for Possible Testing Approaches

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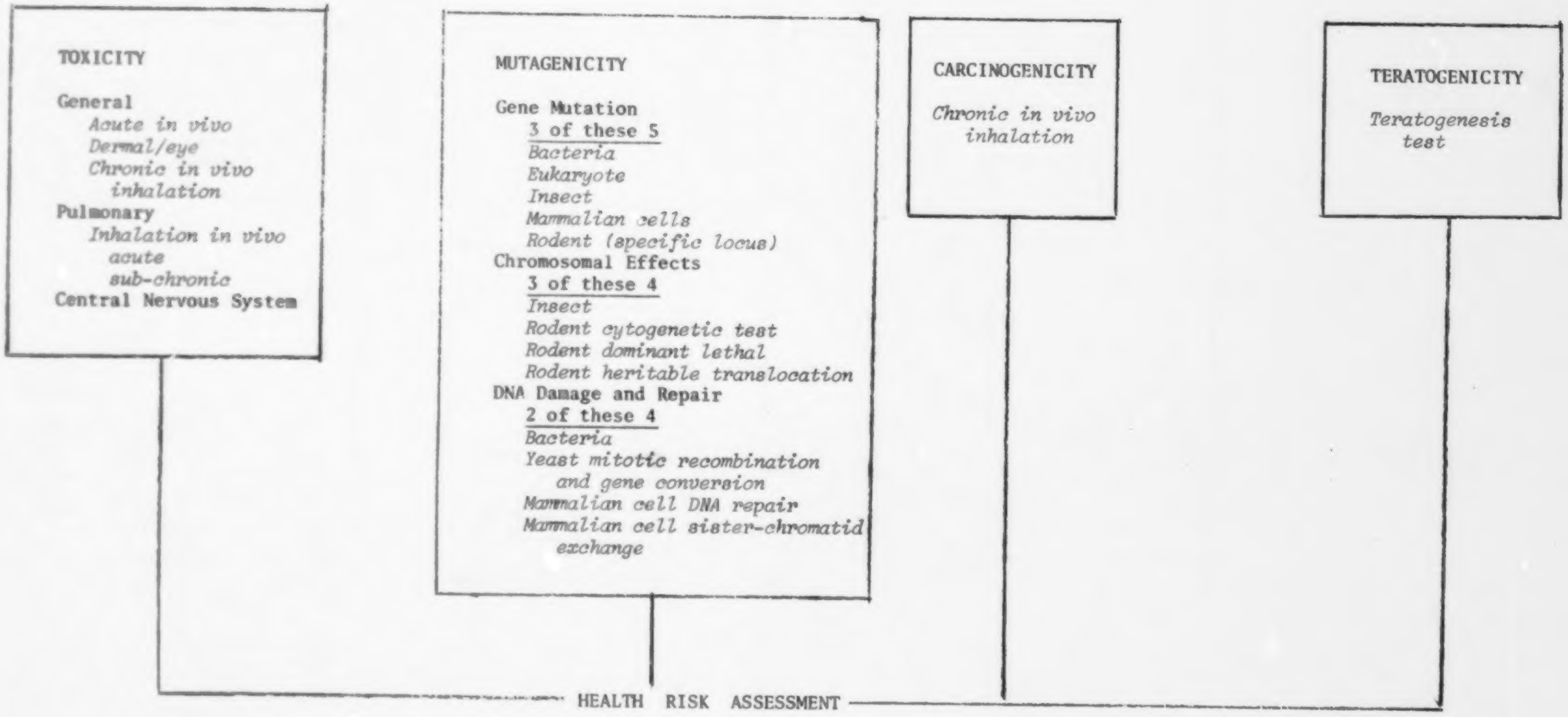
TOXICITY
General
Acute in vivo
Dermal/eye
Chronic in vivo inhalation
Pulmonary
Inhalation in vivo
acute
sub-chronic
Central Nervous System
MUTAGENICITY
Gene Mutation
3 of these 5
Bacteria
Eukaryote
Insect
Mammalian cells
Rodent (specific locus)
Chromosomal Effects
3 of these 4
Insect
Rodent cytogenetic test
Rodent dominant lethal
Rodent heritable translocation
DNA Damage and Repair
2 of these 4
Bacteria
Yeast mitotic recombination and gene conversion
Mammalian cell DNA repair
Mammalian cell sister-chromatid exchange
CARCINOGENICITY
Chronic in vivo inhalation
TERATOGENICITY
Teratogenesis test
HEALTH RISK ASSESSMENT

Figure 1 . *AN EXAMPLE OF A MATRIX APPROACH TO TESTING*

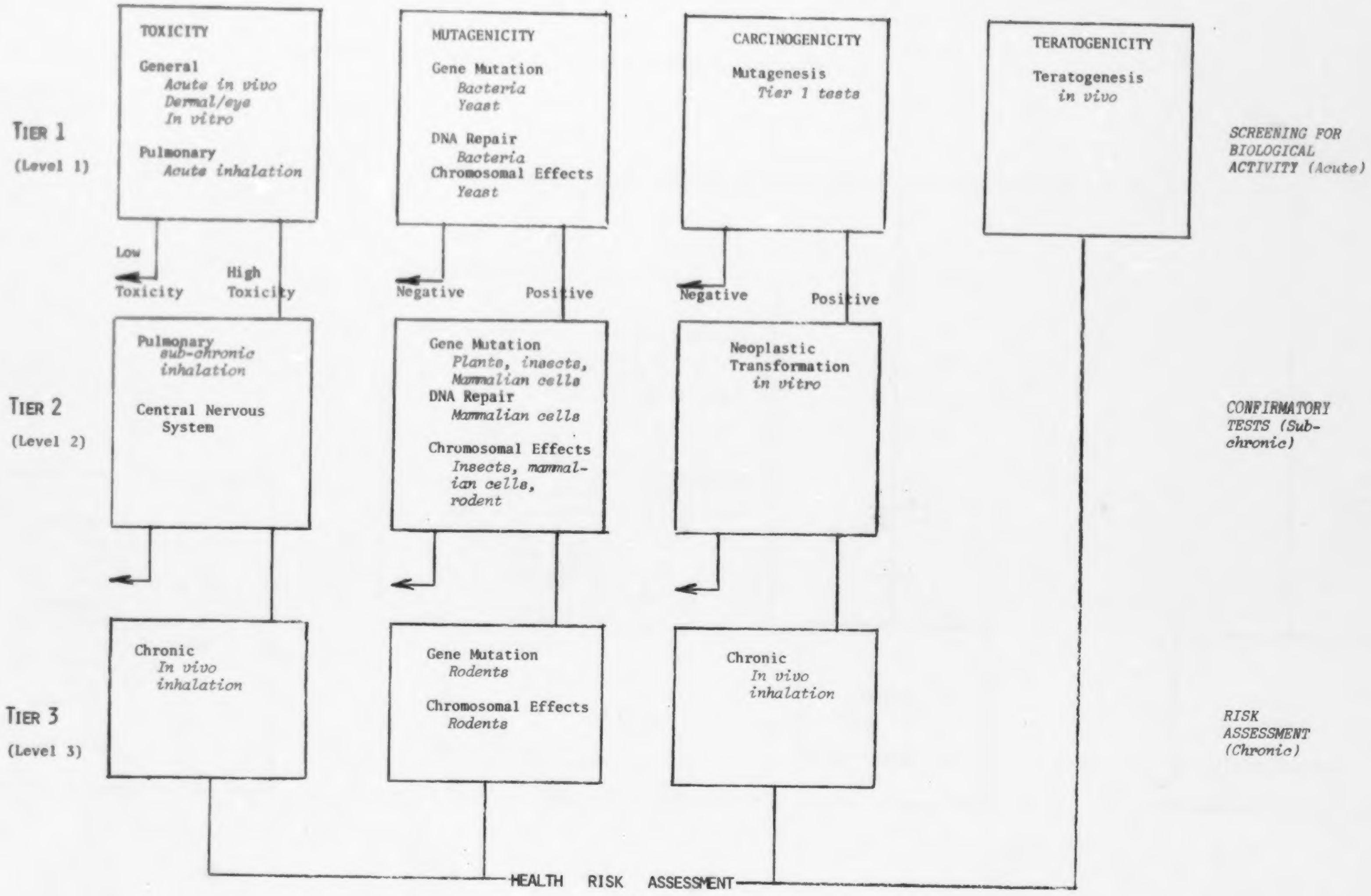
TIER 1
(Level 1)
TOXICITY
General
Acute in vivo
Dermal/eye
In vitro
Pulmonary
Acute inhalation
MUTAGENICITY
Gene Mutation
Bacteria
Yeast
DNA Repair
Bacteria
Chromosomal Effects
Yeast
CARCINOGENICITY
Mutagenesis
Tier 1 tests
TERATOGENICITY
Teratogenesis
in vivo
SCREENING FOR BIOLOGICAL ACTIVITY (Acute)
Low Toxicity
High Toxicity
Negative
Positive
Negative
Positive
TIER 2
(Level 2)
Pulmonary
sub-chronic
inhalation
Central Nervous System
Gene Mutation
Plants, insects, Mammalian cells
DNA Repair
Mammalian cells
Chromosomal Effects
Insects, mammalian cells, rodent
Neoplastic Transformation
in vitro
CONFIRMATORY TESTS (Sub-chronic)
TIER 3
(Level 3)
Chronic
In vivo
inhalation
Gene Mutation
Rodents
Chromosomal Effects
Rodents
Chronic
In vivo
inhalation
RISK ASSESSMENT (Chronic)
HEALTH RISK ASSESSMENT

FIGURE 2. AN EXAMPLE OF AN HIERARCHICAL APPROACH TO TESTING.

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